

GENE ANALYSIS USING OLIGONUCLEOTIDES

by

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Abstract

A method has been developed utilising the specific hybridisation properties of oligonucleotides, to detect single base changes in DNA using a sandwich-assay. The method is based on the covalent immobilisation of one oligonucleotide (capture probe) to a solid support resin and the use of a second ^{32}P labelled oligonucleotide (detector probe) free in solution. In the presence of complementary target DNA both probes will hybridise, resulting in the resin being radioactively labelled. When exposed to stringent hybridisation and washing conditions, only an exactly matched detector probe will remain bound to the target DNA. This relies on the property that mismatched sequences are destabilising to the hybrid complex and will demonstrate a reduced melting temperature under stringent conditions. The genotype of the target DNA can thus be established by running two assays in parallel, with labeled oligonucleotide probes specific for the normal and the mutated coding sequence.

Using the sickle cell condition of the beta-globin gene as a model system, a sandwich assay has been optimised for the genotypic analysis of small (< 200 ng) samples of human genomic DNA. Under optimum hybridisation conditions a detection limit of 1 femtomoles target DNA was found. The analysis of single copy genes (attomolar concentrations of target DNA) using this assay was possible with the application of the polymerase chain reaction. Preliminary work has been carried out to adapt this assay for the detection of two frequently occurring base mutations associated with the alpha-1-antitrypsin gene and correlated with the Z and S Pi types. Efforts have also been made to adapt this assay for a non-radioactive labeling strategy.

The sandwich assay was tested on clinical blood samples from patients of all three genotypes to the sickle condition. Disregarding

the time involved in preparing the assay constituents, the test could be performed in under three hours. As DNA can be amplified from nearly crude blood using the polymerase chain reaction, the complete test could be carried out in less than one day.

"In every field there are a few simple questions which are highly embarrassing because the debate which for ever arises around them leads only to perpetual failure and seems consistently to make fools of the most expert."

E.Erikson

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ABBREVIATIONS

A, C, G, T	Adenine, cytosine, guanine, thymidine
dA, dC, dG, dT, dU	Adenosine, cytidine, guanosine, thymidine, uridine deoxyribonucleotide
2-aminoadenine	1 (2,6-diaminopurine, D)
alpha-1-antitrypsin	α -1-antitrypsin (Mz, Z alleles and Ms, S alleles)
AIDS	Acquired immunodeficiency syndrome
AMP	Adenosine-3', 5'-monophosphate
ATP	Adenosine ribonucleotide triphosphate
dATP, dCTP	Adenosine, cytidine deoxyribonucleotide triphosphate
dGTP, dTTP	Guanosine, thymidine deoxyribonucleotide triphosphate
APTE	Aminophenylthioether
B	Base (appears as a symbol in diagrams)
beta-globin	β -globin gene (AA:homozygous normal, AS:heterozygous for the sickle mutation, SS:homozygous for the sickle mutation)
bp	Base pair
BPB	Bromophenol Blue
BSA	Bovine serum albumin
°C	Degrees Celcius
CAP	Calf intestinal alkaline phosphatase
Carbodiimide (CMC)	1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluene sulfonate
Ce	5-methyl-N4, N4-ethanocytosine
CNBr	Cyanogen bromide
CPG	Controlled pore glass
uCi, mCi, Ci	Micro, milli, Curie
DBM	Diazobenzylloxymethyl
DCA	Dichloroacetic acid
DCE	Dichloroethane
DEAE	Diethylaminoethyl
DITC	p-phenylene diisothiocyanate
DMSO	Dimethylsulphoxide
DMF	N, N-Dimethylformamide
Dmtr	Dimethoxytrityl
dN	Any one of the four deoxyribonucleotides (G, C, A, T)
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
dNTP	Any one of the four deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, dTTP)
dpm	Disintegrations per minute
DPTE	Diazophenylthioether
DTT	Dithiothreitol
<u>E. coli</u>	<u>Escherichia coli</u>
EDTA	Ethylenediaminetetra-acetic acid
Factor VIII	Antihemophilic globulin
Factor IX	Plasma thromboplastic component
FPLC	Fast protein liquid chromatography
G av	Average gravitational force
pg, ng, ug, mg, g	Pico, nano, micro, milli, gram
Hbf 5	Recombinant plasmid containing the normal beta-globin gene insert
Hbl-S	Recombinant plasmid containing the sickle mutated beta-globin gene insert

Hb2-S	Recombinant plasmid containing the sickle mutated beta-globin gene insert in a pUC 13 vector
HBV	Hepatitis B virus
HEPES	N-2-hydroxyethyl piperazine N'-2-ethane sulphonic acid
HPLC	High pressure liquid chromatography
hr, hrs	Hour, hours
HRP	Horse radish peroxidase
IPTG	Isopropylthio-beta-galactoside
kb	Kilobases (1000 bases)
kbp	1000 base pairs
linker	Single stranded sequence, acting as a bridge between two complementary DNAs
ul, ml, l	Micro, milli, litre
luminol	5-Amino-2,3-dihydro-1,4-phthalazinedione
mer	oligomer
MES	2(N-morpholino)ethane sulfonic acid
nm, um, mm, cm	Nano, micro, milli, centi, metre
min	minute
uM, mM, M	Micro, milli, molar
amol, fmol, pmol	Atto, femto, pico,
nmol, umol, mmol	Nano, micro, milli, mole
mononucleotide	Monodeoxyribonucleotide oligomer
mRNA	Messenger Ribonucleic acid
MSNT	1-(Mesitylenesulphonyl)-3-nitro-1,2,4-triazole
NMR	Nuclear magnetic resonance
noise	Background
nr.	Number
one-step reaction	A single hybridisation reaction
O.D.	Optical density
OPD	O-phenylenediamine dihydrochloride
32 P	Phosphorus-32
PCR	Polymerase chain reaction
Phosphoramidite	3'-[(2-cyanoethyl)-(N,N-diisopropyl)] phosphoramidite
PKU	Phenylketonuria
pleomorphic	Occurring in more than one form, polymorphic
poly A	Single stranded DNA homopolymer complex
RFLP	Restriction fragment length polymorphism
RNase	Ribonuclease
rpm	Revolutions per minute
SA-HRP	Streptavidine Horseradish peroxidase conjugate
SDS	Sodium Dodecyl Sulfate
signal	Radioactive signal, emitted from a labelled probe
spacer-arm	Oligomeric spacer [poly (dT or dU)] between 10-20bp
s, as	Sense and anti-sense strand
ss, ds	Single, double strand
TEA	Triethylamine
TEMED	N,N,N',N'-tetramethylethylenediamine
TBE	Tris-borate electrophoresis buffer
Td	Dissociation temperature
Tm	Melting temperature
t RNA	Transfer ribonucleic acid
two-step reaction	Two hybridisation reactions
UV	Ultraviolet
X-GAL	5-bromo-4-chloro-3-indolyl-beta-D-galactoside

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Introduction

Advances in molecular biology and biotechnology have lead to many exciting possibilities for DNA diagnosis. The developments have altered many aspects of modern medicine including the pre-and postnatal analysis of genetic disease, also the analysis of infectious and acquired diseases (for example, malignancies). These developments have, to a large part, been stimulated by the increasing awareness of infectious disorders, and the demand for carrier detection of many inherited diseases.

Only very recently, however, are clinical laboratories considering DNA probes as routine diagnostic reagents. This seems surprising when one considers that the potential applications of the DNA probe were elucidated more than 30 years ago with the discovery of the double helical structure of nucleic acids by Watson and Crick. The main reasons for this delay in accepting DNA probes as useful tools have been the limited availability of non-radioactive techniques, the poor methods of sample preparation, the lack of experience with "dirty" clinical samples and a lack of automation.

For DNA diagnostics to be taken up on a routine basis, methods have to be developed which are simple, inexpensive, sensitive, specific in detection, and reliable. Furthermore, any method developed needs to be a technique adaptable for the diagnosis of any other newly found defects.

Oligonucleotides have established themselves as useful tools for the detection of single base mutations. Their properties provide a new means of diagnostic accuracy that relies on the use of DNA sequence information. The development of detection methods using oligonucleotides will not only open up the possibility of analysing many inherited disorders, but may also further other developments such

as:improved techniques in research,in the detection of pathogenic organisms,in forensic science,plant breeding and oncology.

1.1 The molecular basis of inherited diseases

The study of human haemoglobins over the past three decades has contributed substantially to the understanding of basic genetic principles and disease.Work carried out by Ingram in 1956 provided some of the first molecular evidence that an inherited condition was related to the production of an abnormal peptide,which in turn was caused by a single amino acid change in the beta-chain of adult haemoglobin.Later it became apparent that the abnormality resulted from a single base change in the DNA.These initial studies of sickle haemoglobin and discoveries surrounding other abnormal haemoglobins are reviewed by Conley (1980).

It has only been through the more recent studies of globin genes from various sources (mouse,rabbit,human) and the application of recombinant technology that we have gained a better understanding of the structure and function of the gene.Many of the basic principles found have provided a substantial contribution to our knowledge on the range of structural disorders that can occur in DNA.Not all structural changes in the DNA are deleterious to the individual,and not all changes lead to the manifestation of inherited disease conditions.

Over 3000 conditions in man are thought to be of Mendelian inheritance and of these only a small percentage have known molecular defects.However,with recent improvements in the technology of foetal diagnosis and genetic diseases over one hundred of these disorders can now be diagnosed in utero (Galjaard,1987).

Congenital disorders can be subdivided into three main

categories. The first group consists of chromosomal aberrations, which occur at an overall incidence of 0.5% in all liveborn progeny. (Galjaard, 1982). Most aberrations occur spontaneously and lead to spontaneous abortions of the foetus. They are characterised by gross structural changes such as deletions, insertions or translocations within or between chromosomes. Also included in this group are changes such as the loss or gain of one or more chromosome. Aneuploidy can be acquired by non-disjunction during meiosis, and can lead to autosomal chromosome disorders such as Down's syndrome and sex chromosomal disorders such as Turner's syndrome. Chromosomal additions can also be acquired through isochromosome formation (the formation of an asymmetrical chromosome) and chromosomal mosaicism (the presence of two chromosomal cell lines differing in constitution).

The second group incorporates the congenital disorders arising as a result of single base mutations in the DNA. The estimated incidence of these mutations is thought to be between 0.2-1.3 % of all live births. (Galjaard, 1982) Both autosomal and sex chromosomes can be the targets of such mutation, although the major incidence is in the autosomal chromosomes. Examples of such base alterations are seen in the sickle mutation of the beta-globin gene (Orkin, 1984) and in the many base alterations found to be associated with various alpha 1-antitrypsin deficiencies (Fagerhol, 1981). This type of mutation can also be acquired postnatally as a result of spontaneous changes in the DNA sequence.

The third group consists of all the changes which occur as a result of multifactorial malformations. The overall incidence of these malformations is by far the largest, accounting for 2-3% of the congenital disorders among liveborns. Disorders in this group are brought about by changes assumed to affect the interaction of multiple genes and multiple gene products. Some of the diseases brought about by

such disorders include spina bifida cystica and cleft lip/palate (Galjaard 1982).

Disorders brought about by single base changes in DNA can be inherited in a Mendelian fashion either as autosomal dominant, autosomal recessive or X-linked abnormalities. Of these mutations, some are considered to be "reoccurring mutations" in the sense that they can appear as spontaneous mutations with measurable frequencies as in Huntington's chorea (Hayden, 1981). The pattern of inheritance, dominant or recessive, is determined by the relative effectiveness of the mutant gene in producing abnormality. New mutations generally remain undetected: deleterious mutations are lost through natural selection pressures.

Defective gene products have been tentatively identified for approximately 400 monogenic disorders (Landegren et al., 1988) and the base mutations involved have been determined for at least 45 of these disorders. A few of these have been listed in table 1.

From studies of the beta-globin gene and its surrounding DNA, it has been found that single base mutations can affect general aspects of gene function: transcription, RNA processing and RNA translation. Many classes of defect can however, result in the same phenotypic expression. According to Orkin and Kazazian (1984) 20 of the 30 different mutations identified as leading to beta-thalassaemia have been found to be single base substitutions.

Single base mutations can result in minor structural and functional abnormalities of a protein, others lead to localised or generalised metabolic disturbances, and still others are associated with severe physical and/or mental handicaps and a marked reduction of life expectancy.

Disease	Gene	Reference
α -1-antitrypsin	α -1-antitrypsin (Z and S allele)	*+ Kidd et al.(1984)
Haemophilia A	Factor VIII	* Antonarakis et al. (1985)
Haemophilia B	Factor IX	* Chen et al.(1986)
Beta-thalassaemia	(over 30 have been found)	Bunn and Forget (1986)
β^0 thalassaemia	β^{39}	+ Orkin and Goff (1981)
β^c & Sickle cell anaemia	β^6	*+ Studencki et al. (1985)
β^+ thalassaemia		* Orkin and Kazazian (1983)
Haemoglobin Köln	β^K	* Horst et al.(1986)
Haemoglobin E	β^E	* Thein et al.(1986)
α -thalassaemia	α globin	* Goossen et al. (1982)
Hereditary persistence of foetal haemoglobin	γ globin	* Collins et al. (1984)
Phenylketonuria	Phenylalanine hydroxylase	x Dilella et al. (1986)
Antithrombin III deficiency	Antithrombin	x Duchange et al (1986)
Cancer	H-ras oncogene N-ras oncogene	* Farr et al. (1988) *

Note: Mutations found as a result of altered restriction recognition sites (*);those detected with oligonucleotide probes (+);those revealed through DNA sequencing (x).

TABLE 1 GENETIC DISORDERS CAUSED BY A SINGLE BASE
MUTATION

The single base mutation disorders used in the present study affect the beta-globin gene, leading to sickle cell anaemia and the alpha 1-antitrypsin gene leading to alpha 1-antitrypsin deficiency. Both disorders arise from the substitution of one base for another (point mutation), both are located on autosomal chromosomes and result in the production of defective gene products.

Sickle cell anaemia is characterised by a single base mutation affecting the sixth codon of the beta-globin gene (on the short arm of chromosome 11), abolishing a site for the restriction endonucleases Mst II and Dde I. It is one of the most common heritable disease in people of African origin (Weatherall, 1985) and is said to have a heterozygotic frequency of 7.3% in the U.S black population (Caskey, 1987). The base substitution with which it is associated is a change of an adenine to a thymidine, leading to a respective change in amino acid sequence from a glutamic acid to a valine. The result is the production of the structurally defective sickle shaped haemoglobin, which can induce severe anaemia. The globin chain product is however still synthesised at an approximately normal rate.

The advances made in uncovering the mutations of the Human globin genes are reviewed by Neinhuis et al (1984), also Orkin and Kazazian (1984).

The alpha-1-antitrypsin gene, located on chromosome 14, is highly pleomorphic: more than 30 different haplotypes have been described (Fagerhol 1981). A deficiency of this gene is often associated with childhood liver disease and/or adult emphysema (Eriksson et al, 1986).

The alpha-1-antitrypsin phenotype, referred to as the protease inhibitor (Pi) type, represents the co-dominant expression of the two parental haplotypes. The most common haplotype in the USA are those of the M-family (the normal haplotype), the S type (frequency, 0.02-0.04), and the Z type (0.01-0.02) (Dykes et al, 1984). Both the Z and the S

genes are of clinical interest, as their recessive inheritance has been strongly correlated with the development of severe disease conditions both in the adult and child (Nukiwa et al, 1986; Cox and Mansfield, 1987). Each of the two mutants code for proteins that differ from the M protein, by a single amino acid. In the S protein, valine 264 replaces glutamic acid 264, whilst, in the Z protein, lysine 342 replaces glutamic acid 342.

Individuals with either of these mutant phenotypes often suffer from alpha-1-antitrypsin deficiency. Those with the homozygous Z pi allele usually experience a more severe deficiency, with a plasma level of 10-15% of the normal level. (Allen et al, 1974) The plasma level of alpha-1- antitrypsin in the heterozygous individuals with the M genotype is about 50-60% of the normal (Fagerhol, 1970). Further details on the molecular physiology and pathology of alpha-1-antitrypsin are given in reviews by Boswell and Bathurst (1985), also by Tetley (1984).

Although the relative frequencies of affected homozygous individuals suffering from either of the disorders described is low, there is a demand to establish specific and efficient tests particularly for prenatal diagnosis, as the carrier frequency of both disorders is high.

1.2 Acquired and infectious disorders

Acquired disorders have been classified as those conditions which arise spontaneously in an individual's life and are often associated with rearrangements in DNA sequence. These rearrangements have been found to occur in familial as well as sporadic forms (Knudson, 1986), and commonly lead to the manifestation of cancers and other malignancies.

Both dominant and recessive modes of cancer inducing

transformations are said to exist. Dominant acting oncogenes are thought to constitute a group of genes totalling 40 at the present time (Bishop, 1987). These induce transformations when introduced into a cell or an animal in a structurally altered form, or when improperly expressed. They are activated by a variety of mutational mechanisms: by point mutations, chromosomal rearrangements and translocations, or amplification of oncogenic sequences (Caskey, 1987).

Recessive oncogenesis is thought to constitute those instances where the tumor cells have lost copies of genetic sequences which appear to act as "tumor repressors" (regulating cell growth). The introduction of one normal chromosome would be expected to correct the phenotype.

The finding that c-myc was activated by translocations involving immunoglobulin sequences on several chromosomes and the c-myc sequences illustrated oncogene activation by chromosomal rearrangement (Bishop et al, 1987). Activation was found to result in enhanced transcription of the c-myc sequences the over expression of which has been associated with Burkitts lymphoma. Leukaemias have also been associated with chromosomal translocations, such as promyelocytic leukaemia (Dayton et al, 1984).

The somatic cell loss of genetic sequence on the other hand has been associated with the autosomal dominantly inherited form of retinoblastoma. It is speculated that the loss of repressor sequences is involved in the transition to neoplasia (Cavenee et al, 1983).

More interesting from the point of view of this study, the activation of some oncogenes have been associated with the appearance of point mutations (Finkel and Cooper, 1986). Specific point mutations in the Ras gene have been implicated in the development of a number of human malignancies, such as colorectal cancers as well as pancreatic (Bos et al., 1987), and haematopoietic cancers (Farr et al., 1988).

This gene contains three protooncogenes, h-ras, k-ras and n-ras. These encode specific proteins that bind guanine nucleotides, possess intrinsic GTPase activity, and are located at the inner surface of the plasma membrane of normal cells. Activating point mutations of the RAS gene are thought to alter the functions and biochemical behaviour of the protein products, which are, in turn, thought to contribute to the manifestation of neoplastic growth (reviewed by Varmus and Bishop, 1986).

It seems likely that further research in this area will reveal other point mutations that convert proto-oncogenes to oncogenes.

Although an immediate use for the rapid diagnosis of these mutations is not apparent, such analysis may assist quantitation of oncogene expression and therefore clinical staging of diseases for therapy selection (Seeger et al., 1985).

Infectious diseases are caused by microbial pathogens which can be transmitted from host to host through a variety of media, such as air, water, food and insects. Their infection into the human host can lead to a number of known disorders, varying in virulence and pathogenicity. Their classification with respect to human diseases usually falls into four categories: the bacterial infections (enterobacteria and non-enterobacteria), sexually transmitted diseases, viral infections (in the blood, in stools, and in tissues), and parasitic infections.

With the increased awareness of the incidence of several diseases such as hepatitis B virus (Lin et al 1987) and acquired immunodeficiency syndrome (Waterson, 1983) the developments for improved vaccines and diagnostic assays have been encouraged. As little or no detectable antigen is produced by these infections, DNA based detection systems have played an important role in the development of

new diagnostic assays.

Also of concern, recently, is the correlation found between cancers and the appearance of viruses in, for example, the papilloma virus and cervical cancer. (Gissmann et al., 1983) This interesting association between infectious disorders and the acquisition of cancer opens the way for further correlation of other viral invasive diseases with cancers.

Unlike inherited disorders, the genetic changes that can result from infectious disorders are only now being elucidated. Viral integration into the host genome, resulting in microdeletions and chromosome translocations, has been observed with respect to the hepatitis B virus (Nakamura et al., 1988). Taking into account the link between viral infection and the manifestation of cancer, one can not exclude the possibility of other structural rearrangements also taking place.

1.3. Diagnosis of diseases, current aspects and classical methods.

Fundamental to the treatment and prevention of any disease is accurate diagnosis. Past diagnostic procedures relied heavily on the manifestation of clinical symptoms and/or family studies. Tests used to determine the levels of metabolic proteins and sugars assisted, for example, in the neonatal screening of phenylketonuria and galactosaemia. Similarly, family studies and extensive counselling were used to reduce the incidence of Tay-Sachs disease.

Although these are still important features of present day diagnosis, progress in immunology and molecular genetics has resulted in new and more accurate methods of determining diseases. These developments have changed many aspects of modern medicine including the awareness of inherited and environmental risk towards disease. The

effects can be seen in the pre- or post natal analysis of genetic diseases, the identification of individuals predispositioned to conditions such as diabetes or coronary heart disease, the diagnosis of infectious diseases, and the classification of malignant disease.

1.3.1 The impact of molecular cloning on the diagnosis of disease

The techniques of DNA and antibody cloning developed over the past decade have probably had the greatest impact on the development of new therapeutic and preventative interventions as well as the diagnosis of diseases. Cloning has made it possible to construct and isolate defined macromolecules with specified functions in order to gain a better understanding of inherited, acquired and infectious diseases.

In gene cloning, a particular sequence of DNA is separated and propagated independently from an organism's genome. Usually this is done by cutting extracted DNA material into small fragments with restriction endonucleases. The fragments are then ligated into vectors that have been cut with the same or compatible endonucleases, and the recombinant DNA is inserted into a bacterial host such as E.Coli.

For the cloning of monoclonal antibodies, a specific committed B cell is separated from all other B cells. Committed cells are those that have been exposed to an antigenic challenge, through infection or immunisation, and ultimately produce the antibody of interest. Cell fusion with myeloma cells then renders these cells immortal, allowing them to grow for prolonged periods in culture.

The availability of cloned products for clinical use was an improvement over the methods involving biochemical purification in several ways. First, cloning provided an inexhaustible and renewable supply of product. Secondly, cloning provided an absolute separation of

molecular elements from other similar elements. Gene cloning, for instance, permitted the isolation of unique DNA sequences or gene products from all other similar elements in the donor species. Thirdly, the sensitivity and specificity of a cloned reagent is limited primarily by the user, rather than by the purity of the reagent itself. Finally cloning is reproductive and has allowed for consistency in experimental work and developments.

Monoclonal antibodies are now commonly used for the rapid identification of clinical isolates and the early diagnosis of many infections by direct testing of clinical specimens. The tests rely on the isolation of a single or a group of monoclonal antibodies that recognise specific epitopes of the pathogen of interest. Other applications of monoclonal antibodies have been in the development of vaccines. New developments have suggested the use of monoclonal antibodies in targeted drug delivery and for providing passive immunisation against pathogens. Their application could also be in the neutralisation or elimination of microbial toxins. The impact of cloning techniques on the diagnosis and treatment of infectious diseases is reviewed by Engleberg et al. (1984)

Monoclonal antibodies have been of limited use with respect to the more cryptic infections, as is displayed by the hepatitis B virus (Brechot et al 1981) where detectable antigens are disguised by antibodies against them. Also many latent infections can not be identified easily using monoclonal antibodies and the opportunities of misdiagnosis due to antigenic variation of a pathogen are great. Furthermore, monoclonal antibodies have limited specificity in detecting genetically inherited diseases or early changes associated with malignant disorders. It is in these areas that cloned DNA sequences have been the better diagnostic reagents.

More recently studies have been directed towards replacing

immunologically produced vaccines with recombinant vaccines. Initial studies have shown recombinant DNA-based vaccines to be as effective as natural vaccines, in inducing protective immunity (Egerton et al., 1987; Just et al., 1987). Already clinical trials have been set up, inoculating infants born to Hepatitis B antigen positive mothers, and adults, with recombinant DNA vaccines (Cadranel et al., 1987; Laukamm et al., 1987).

Recombinant vaccines ultimately would not only be less costly to produce, but might also provide a more effective means of generating nontoxic and avirulent vaccines. By using recombinant techniques, it may in future be possible to identify specific antigenic domains and surface exposed epitopes of a whole range of pathogens thus enabling the development of new vaccines against them.

1.3.2 The methods used in diagnosing diseases with DNA probes

Fundamental to DNA diagnostics is the principle that single stranded DNA will re-form hydrogen bonds when it contacts an exactly complementary single strand of DNA. Mis-matched strand formation is unstable and will not occur under stringent hybridisation conditions. This reaction is so specific that even small probes 15-20 bases long, can identify relevant sequences in the presence of 10^9 nucleotides worth of "irrelevant" target sequences. Considering that the human genome contains a total of 3×10^9 base pairs of DNA, of which only 3-5% encode gene sequences, this specificity is sufficient for the unambiguous detection of any target DNA or RNA sequence of clinical importance (reviewed by Lowe, 1986).

It is this property of nucleic acid hybridisation that is the basis for the specificity of all DNA based detection

techniques. Single-stranded probes can be generated by cloning DNA sequences from the DNA or RNA of an isolated pathogen or from DNA informative to the disease being analysed. More recently with the possibility of oligonucleotide synthesis the same probes can be made without the need for cloning, provided the DNA sequence is known or a short stretch of protein sequence has been characterised.

To identify and quantify the DNA probe it is labelled either with radioactive or non-radioactive nucleotides, hybridised to immobilised target DNA and identified either colorimetrically or by autoradiography.

The process of DNA based diagnosis can generally be divided into three parts: 1) sample preparation, 2) molecular hybridisation, and 3) hybrid detection.

1.3.2.1 Sample preparation

Sample preparation includes those manipulations required to present the target DNA, in a clinical sample, to a labelled probe, in a form that permits molecular hybridisation between the two. Typically this involves the purification of DNA and RNA away from macromolecules that interfere with molecular hybridisation. Sample preparation is still the most time consuming, expensive and difficult step in gene diagnosis. Once isolated the target DNA is restriction endonuclease digested to lengths suitable for further manipulations without damaging the sites of genotypic interest.

The DNA or RNA sequences from clinical samples for gene analysis are usually obtained from one of three sources: from peripheral blood cells, from foetal cells and from cells in tissue samples.

DNA for carrier detection of inherited disease in children and adults may be obtained from peripheral blood leucocytes. From blood

leucocytes, yields of up to 200ug DNA can be extracted from 10-20mls blood.

Prenatal diagnosis requires tissue of foetal origin, which can be obtained in one of several ways. Amniocentesis, performed in the second trimester, can provide foetal cells present in the amniotic fluid (reviewed by Robinson, 1985). From these cells DNA can be extracted either directly (Chang and Kan, 1982), or the cells can be cultured for two to three weeks to increase the yields (Gosden, 1983). Alternatively chorionic villus sampling can be carried out, involving the biopsy of foetal trophoblastic tissue, through transcervical aspiration (Williamson et al, 1981) or using rigid forceps (Monni et al., 1987). Trophoblastic tissue establishes itself two to three weeks after fertilisation and undergoes atrophy after twelve weeks. Generally sampling of this tissue is therefore performed in the first trimester. This technique may yield 10-50ug of DNA per biopsy, with minimal maternal contamination of genetic material (Martinville et al, 1984).

Two less used methods can also be used to obtain foetal cells. One is by a method of placental aspiration, (Kan et al, 1974) removal of foetal blood cells from the placenta, the other is by foetoscopy, (Hobbins and Mahoney, 1974) removal of foetal blood from the chorionic plate or the umbilical vein. Both methods are less desirable as they carry a high risk of foetal mortality, (Alter, 1981) as well as maternal blood contamination.

Sampling procedures for the detection of acquired and infectious disorders generally uses those tissues affected by the disease for analysis. Any tissue from which DNA can be prepared either directly or indirectly through tissue culture is suitable, such as whole blood, muscle biopsies, and bone marrow biopsies. Similarly any specimen whether urine, faeces or other "exudates" containing the pathogen can

be used for DNA extraction.

Recombinant techniques (cloning) are used to prepare the unique probe that will identify either the infectious pathogen or the genetic defect at the DNA level. For the analysis of infectious disorders, the DNA probe is prepared either directly from sequences derived from the pathogen (Chisaka et al., 1986) or from the sequences of related pathogens. Thus probes may be developed that are either exclusive to one species of bacterium or viral strain or common to all species in a given bacterial genus or viral class (Franzen et al., 1984). More recently probes can be synthesised as oligonucleotides whose sequence is derived either from DNA, RNA or protein sequence data unique to the pathogen (Lin et al., 1987).

Probes for gene analysis require the knowledge of the underlying genetic defect. Several approaches are used for generating gene probes. One method used when the expression of a defective gene is at high levels, is to extract and purify the mRNA in order to enzymically generate cDNA. This can then be used directly as a probe or for further cloning strategies. Another method involves isolating mRNA sequences from a cloned library of total mRNA sequences constructed from the tissues in which the gene is known to be expressed. The mRNA is identified through the specific hybridisation of an oligonucleotide probe, which is designed around the knowledge of DNA or protein sequence data (Reyes and Wallace, 1983). Again, cDNA is generated from the mRNA. Both sources of cDNA can be purified from the complementary mRNA prior to use as probes (Szybalski et al., 1985).

Although our DNA sequence data is still limited, future availability of the complete human genome should make it possible to identify more human genes, and sequences, of diagnostic value in a wide variety of disease states. These advances may also encourage the development of new methods for synthesising DNA probes.

1.3.2.2 Hybridisation

Hybridisation of both probe and target sequences is performed in a variety of ways. The most established and widely used techniques are the direct hybridisation methods of Southern (Southern, 1975) and dot (Kafatos et al., 1979) or slot blotting, as is the equivalent technique for RNA known as Northern blotting. In these hybridisation reactions the target DNA is immobilised to a solid membrane support such as nitrocellulose or nylon, and hybrid formation to the probe is measured as labeling of the filter. Prior to immobilisation the DNA is restricted, through endonuclease digestion, and is denatured, or is left whole as is the case for RNA. Southern or Northern blotting enables the analysis of electrophoretically separated fragments of DNA, whereas dot blotting examines mixtures of unpurified target nucleic acids. Under stringent conditions the single stranded, labelled probe DNA is then hybridised to the target DNA, revealing the presence of complementary sequences.

a) Advances made in hybridisation techniques

Techniques have been developed as improvements to the more classical method of filter-hybridisation. Some of these improvements have been in the transfer of target material to the solid support. Southern blotting has the major drawback that large DNA fragments (>10 kb) are not transferred efficiently. Furthermore small fragments (<200 bp) do not bind well to the most commonly used support matrix, nitrocellulose.

Depurination (acid nicking) of large fragments has been found to increase transfer efficiency but is a reaction difficult to control

(Wahl et al., 1979). Electroelution of the DNAs from agarose or acrylamide gels, using a combination of low and high ionic strength buffers under a high voltage gradient, has since been found to transfer fragments as small as 47bp and as large as 49kb (Zassenhaus et al., 1982).

New solid support membranes have been developed to immobilise the DNA more efficiently. The unique properties of nylon membranes have allowed for a dramatic improvement in the capillary transfer of DNA restriction fragments from agarose gels (Reed and Mann, 1985). These unique properties relative to nitrocellulose are considered to be: a greater mechanical strength, a higher capacity for nucleic acids, the ability to bind smaller oligonucleotides, a stronger retention of bound nucleic acids, and the ability to permit multiple re-probing of filters (Cannon et al., 1985). In addition to this it has been reported that ultra violet radiation catalyses the covalent attachment of nucleic acids to these membranes.

Numerous nylon membranes have been developed suited to immobilising large (1000bp) and small DNA fragments (>500bp). Zeta probe for instance has been claimed by the manufacturers (BRL) to be capable of immobilising oligonucleotides as small as 10 nucleotides long. Another advantage of Zeta probe that is claimed is that it can be used in the rapid electrophoretic transfer of DNA and RNA, which is exactly reproducible and quantifiable in terms of volt-hours.

Stark and his colleagues (1979) demonstrated that by using diazobenzyloxymethyl-paper, single-stranded DNA and RNA fragments of any size could be transferred more efficiently, from the agarose-acrylamide gels (Wahl et al., 1979).

Advances have been made in the electrophoretic separation techniques required for Southern transfer. It was found that electrophoresis in low percentage agarose gels or in acrylamide gels

eliminated the difficulties of separating large (>10kb) and small (<1000bp) DNA fragments respectively. Very large fragments (>30kb) are however not easily resolved on agarose gels. A recent development of electrophoresis by periodic inversion of the electric field, "pulse-field" electrophoresis, has made it possible to selectively resolve DNA's in the size range of 15 to >20 000 kb. (Clark et al., 1988).

Efforts have been aimed at establishing hybridisation assays without a filter support system. Hybridisations have been performed directly to the dried down gel matrix, used in separating the DNA target fragments (Purello and Balazs, 1983). Methods have used the gel matrix as a solid support for dot-blot hybridisations, whereby the DNA is embedded in the gel directly, prior to drying the gel down under vacuum (an adaptation of the spot-blot assay by Cunningham, 1983). DNA has been immobilised to polystyrene microtitre wells (Nagata et al., 1985), and to microgranular support matrices such as Sephadex, Sephacryl and Sepharose (Bunemann et al., 1982 a)). Even a fluorocarbon solid support system has been suggested to be suitable for DNA immobilisation and hybridisation reactions (Eveleigh, 1988).

Recently E.I. du Pont de Nemours, & Co., Inc., have developed fluorocarbon liquid and solid support affinity supports, which have been used primarily to immobilise perfluoroalkylated protein ligands such as horseradish peroxidase and urease. However, by labelling DNA with immersion adsorbed perfluoroalkylated calf thymus histones, strong and irreversible binding to the surfaces of solid gas permeable fluorocarbon membranes has also been achieved. This new biospecific support has the following unique advantages; that it can be sterilised, ligands can be easily bound, it has demonstrated excellent kinetics and mechanical stability, it is associated with low ligand leakage, minimal non-specific binding of contaminants, and that it is

chemically inert and reusable.

Solution hybridisation techniques have been developed for the hybridisation of RNA probes to RNA target RNA (Pellegrino et al., 1987) as well as the hybridisation of DNA probes to DNA target (Edelstein, 1986). The first method relies on the activities of the enzyme RNase A, which destroys all unhybridised strands of RNA after hybridisation has been allowed to take place. The hybrids can then be precipitated on to membranes filters for quantitative analysis. The second method relies on the differential binding affinities of double and single stranded DNA to hydroxylapatite. The hybrid complexes are preferentially immobilised and the results indicating the presence or absence of a sequence.

Another quantitative assay for the detection of any RNA or DNA sequence obviating the need for sample immobilisation has been suggested by Vary et al. (1986). The method involves the displacement of a labelled nucleic acid (signal strand) hybridised to the sample DNA, by a longer unlabelled "probe strand". The method is outlined in figure 1. and is known as the "strand-displacement assay" (Ellwood et al., 1986). A separation step is then performed to distinguish between hybridised signal strands and those free in solution. This step is based on the conversion of the polyadenylate tracts (attached as a label) of the free signal probe to ATP by enzyme activity. The ATP is then quantified by a bioluminescence assay.

An alternative strand-displacement assay described by Vary (1986, 1987) uses similar methods to displace an immobilised signal:probe complex with complementary target DNA. Displacement events were quantified by determining the amount of signal DNA present in the supernatant.

Crosslinking reagents used to maintain hybrid stability during separation and/or detection by covalently bonding probe and target DNA

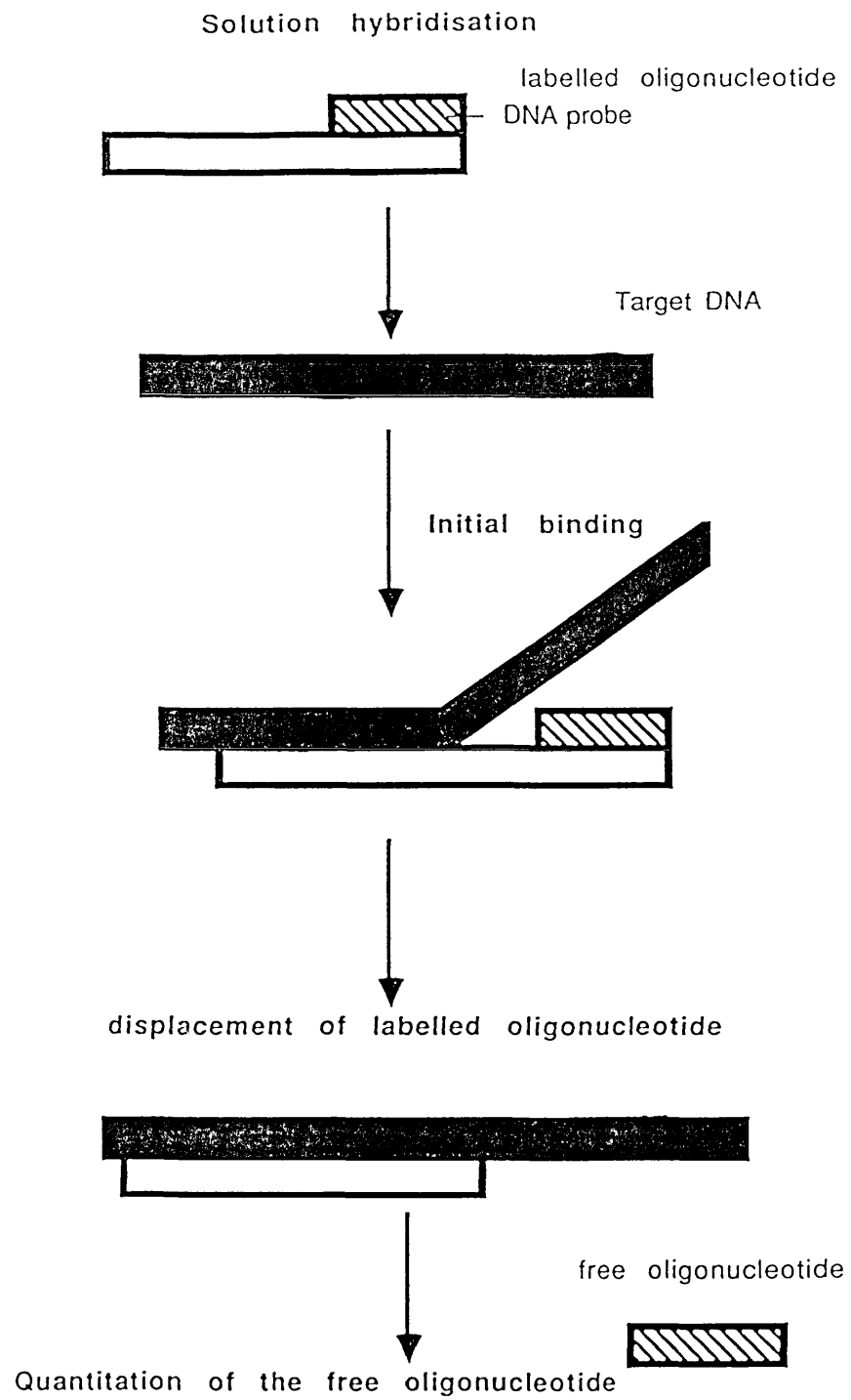


FIGURE 1:STRAND DISPLACEMENT DNA PROBE ASSAY
(Ellwood et al.,1986)

have been found. The bifunctional crosslinking reagent psoralen has been used in this way (Yabusaki et al., 1985). Covalent adducts are formed (Kanne et al., 1982) between the psoralen molecule and adjacent pyrimidine bases on opposite strands of a double helical segment of DNA or RNA, upon exposure to long-wavelength UV radiation (320-400nm). The reaction takes place primarily between thymidines in DNA and uridines in RNA and is photoreversible by exposure to shorter wavelength UV light (typically 254nm) (Cimino et al., 1986).

Another approach has been to design modified polynucleotides that bind irreversibly to their complementary sequence via covalent crosslink (reviewed by Knorre and Vlassov, 1985). One report details the synthesis of oligonucleotides containing the modified base 5-methyl-N⁴,N⁴-ethanocytosine (Ce). Oligomers containing the Ce base form a cross-link when hybridised to their complementary sequence. Hybridisation triggers alkylation between the bases, however the rate of cross-link formation is slow (Webb and Matteucci, 1986).

b) Controlling the specificity and rate of hybridisation

The stringency of hybridisation is regulated by performing the reaction as close as possible to the melting temperature (T_m) of the resulting hybrid complex. By allowing hybrid formation to occur near the melting temperature of DNA duplex one is able to derive the optimal conditions for discriminating between perfect and imperfect hybrids. The melting temperature is affected by base composition (% G + C) and length of the shortest chain in the duplex (n). Other criteria such as pH, ionic composition and concentration (M , in mol/liter) of the hybridisation buffers, as well as the concentration of helix destabilising agents such as formamide (McConaughy et al., 1969), urea (Dutton and Chovnick, 1987) or chaotropic salts (for example NaI,

Hamaguchi and Geiduschek, 1962) also affect the melting behaviour. Meinkoth and Wahl (1984) found the following equation derived from analysing the influence of these factors on hybrid stability:

$$T_m = 81.5^{\circ}\text{C} + 16.6 \log M + 0.41 (\% \text{ G} + \text{C}) - \frac{500}{n} - 0.61 (\% \text{ formamide})$$

This equation applies to the hybridisation of probes longer than 50bp. Oligonucleotide hybridisations show decreased hybrid stability. Generally hybridisations with probes greater than 35 bases long are performed at low temperature ($<50^{\circ}\text{C}$) in the presence of agents that destabilise probe:target hybrids (for example, formamide or chaotropes) or at high temperature ($60-70^{\circ}\text{C}$) in their absence.

The advantage of using high temperatures is the increase in hybridisation rate, which accelerates until the melting temperature is reached. The advantage of low temperature systems is convenience and the preservation of target structure. High temperatures for instance have been found to degrade DNA:RNA hybrid structure (Kourilsky et al., 1970).

Irrespective of the temperatures used, the hybridisation rate is still strongly affected by the membrane bound target. Several ways of accelerating this rate have been suggested. One way is to decrease the hybridisation volume to drive the reaction with a vast excess of probe (Wetmur, 1975). Additives such as dextran sulphate (Wahl et al., 1979) and polyethylene glycol (Amasino, 1986) have been shown to be effective in this way, by excluding the probe molecules from the volume occupied by the polymer, resulting in an apparent increase in probe concentration (Chang et al., 1974).

Recently it has been shown that the kinetics of the hybridisation

reaction can be improved through the incorporation of the Rec A protein and the single-stranded DNA-binding protein, into the hybridisation solution. These additions, in the presence of ATP, accelerate the rate of reaction considerably (Zapolski et al., 1985). Another approach to improving hybridisation rates has been to look at other methods and chemistries as well as alternative support systems for immobilising target DNA. Ultimately the optimum conditions will be ones utilising liquid hybridisation techniques in very small volumes, where both target and probe DNA are freely diffusable.

1.3.2.3 Hybrid detection

In order to differentiate between the signals obtained from hybridised probes and those from unbound probes during filter hybridisations, stringent washing manipulations are carried out. These manipulations not only remove non-specifically bound probes but also remove background binding of probe (noise) from the support system itself. Unsuccessful removal of unbound probe will limit the sensitivity of detection. To reduce the background, blocking agents are used during hybridisation. Included in those reported are the following; heparin (Singh and Jones, 1984), Salmon sperm DNA, yeast tRNA, poly (A), Bovine serum albumin, Denhardt's solution (Denhardt, 1966) and milk powder.

Various substances have been used as labels for DNA probes. Traditionally nucleic acids have been labelled with radioisotopes, however recent developments have focused on alternatives, primarily due to the associated problems of waste and stability of the probes. Also of concern have been the aspects of safety in handling radioisotopes.

Many of the labels (haptens) used for DNA probe assays have been those also used for immunoassays (for example biotin, enzymes and fluorescent groups). However, as yet, no one labelling strategy has emerged as an ideal substitute for radioisotopes. Matthews and Kricka review (1988) the recent labelling strategies that have emerged, concluding that few of the systems that have emerged fulfil the following properties required of an ideal labelling system:

- 1) It needs to be easily attached to DNA,
- 2) detectable at low concentrations, using simple instrumentation,
- 3) produce a signal which is modulated when the labelled probe is hybridised to its complementary sequence (thus facilitating the development of non-separation DNA probe assays),
- 4) stable at elevated temperatures.

Generally a labelling strategy is considered to be satisfactory if the probe can detect a unique gene in a sample of genomic DNA. Each label has its own detection limits and it is this that will determine the sensitivity of a probe assay system. Most methods of labelling non-synthetic probe DNAs with radioisotopes ie. nicktranslation (Rigby et al., 1977) and oligolabelling (Feinberg and Vogelstein, 1983, 1984) fulfill this criteria, (review, Cunningham and Mundy, 1987). Hybridisation assays using ^{32} P-labelled probes can detect down to 1.6 amol and 5 amol respectively (Langdale and Malcolm, 1985), but require long exposure times to autoradiography at these DNA concentrations.

DNA probe assays based on non-isotopic labels which provide signal amplification (such as enzymes), are likely to be more sensitive than assays using labels which provide only a single signal per label (such

as fluorescein, Smith et al., 1985). Only recently has data appeared involving enzymes as labelling agents which has suggested that even smaller limits of detection may be reached with enzymes such as alkaline phosphatase (Jablonski et al, 1986) and horse radish peroxidase (Figueiredo and Malcolm, 1986) using colorimetric and chemiluminescent substrates respectively.

Data handling is an important point to consider with respect to diagnosis and gene detection as it is at this stage that hybridised probe values are converted into clinically meaningful parameters. Problems in data handling arise during quantitative gene diagnosis, (ie. when is a measurement medically positive?) as well as during qualitative analysis (ie. is the presence or absence of a fragment a real effect or is it a result of experimental error?). It is this aspect which has cast suspicion on many of the claims made with respect to probe sensitivity and experimental findings. On the other hand the greatest opportunities in gene diagnosis probably lie in this area.

1.3.3 The determination of genotype using DNA probes

Although a considerable amount of genotypic analysis now involves filter hybridisations using specific target DNAs and probes, most initial diagnosis still relies on methods such as: Karyotyping of the chromosomal complement, determining blood constituents and concentrations through isoelectric focusing or enzyme assays. With respect to prenatal diagnosis and the carrier detection of biochemically less understood disorders, unexpressed disorders or disorders resulting from very small chromosomal alterations, DNA probe analysis has now become indispensable.

Once DNA probes are available the genotype can be determined in

one of two ways; either by indirect analysis using gene linked polymorphisms in restriction enzyme recognition sites, resulting in restriction fragment length polymorphisms (RFLP), or by direct analysis of the mutant gene (review Lowe, 1986).

Due to the polymorphic nature of the human genome restriction fragment analysis only becomes meaningful when backed up by an initial study of the RFLP pattern of several families. These families are ones in which individuals with the disease, normals and carriers are identified (Bolhuis et al., 1987). Furthermore, cloned gene fragments may not always be available for many diseases in which case RFLP analysis is only useful when the restriction site (detected by the available DNA probe) is significantly linked to the gene of interest. Close linkage is also of importance in order to minimise the risk of misdiagnosis, by reducing the likelihood of meiotic crossing over events (Camaschella et al., 1988).

Using this method of diagnosis ten common restriction site polymorphisms in the human phenylalanine hydroxylase locus have now been identified (Lidsky et al., 1985). This has made it possible to identify 90% of the PKU families in the Caucasian population prenatally. Using linked RFLP markers and cloned DNA fragments which can identify these markers, prenatal diagnosis has also been possible in many cases of Duchenne muscular dystrophy (an X-linked lethal disorder, 30% of the cases arising from new mutations) (Wieacker et al., 1983).

Direct analysis of a disorder is performed when the exact mutation responsible for the genetic alteration is known. Generally these mutations are point mutations in genes for which the sequence is also known. Three methods of direct analysis have evolved. The first is applicable when the mutation occurs within the recognition site for enzymes, as in sickle cell anaemia. Probe hybridisation to this site or

sites close in proximity will reflect the change in restriction fragment length, through changes in electrophoretic mobility.

An alternative method relies on the specific hybridisation properties of oligonucleotides to distinguish between single nucleotide differences under the appropriate hybridisation conditions. By using one oligonucleotide complementary to the normal and one to the mutated sequence as probes, differential hybridisation methods can establish the genotype of the target DNA (see figure 2.). This procedure has allowed for a number of mutant alleles to be distinguished from the normal alleles, for use in carrier detection and prenatal diagnosis. It has also assisted in the detection of single base mutations resulting in new variants of established phenotypes (Klasen et al., 1987).

A third technique although not used extensively has involved the formation of RNA:DNA hybrids. When treated with the enzyme ribonuclease A even a single mismatch in hybrid formation will result in a cut. The lengths of fragments that are generated by this treatment indicate the position of a mutation site (Myers et al., 1986). This method also permits the genotypic analysis of known mutation sites.

Direct analysis has not only involved the detection of specific point mutations to establish genotype. Recent immunological work has used oligonucleotide probes as markers for establishing immunoglobulin gene rearrangements often important for establishing B-cell lineages, which can often be an indicator of malignancy (Cleary et al., 1984). Other work has shown how oligonucleotides coding for sequences of repetitive DNA can also act as markers characterising a particular individual's phenotype through a very specific RFLP pattern. This has helped document engraftments in bone marrow transplants, by differentiating between donor and recipient

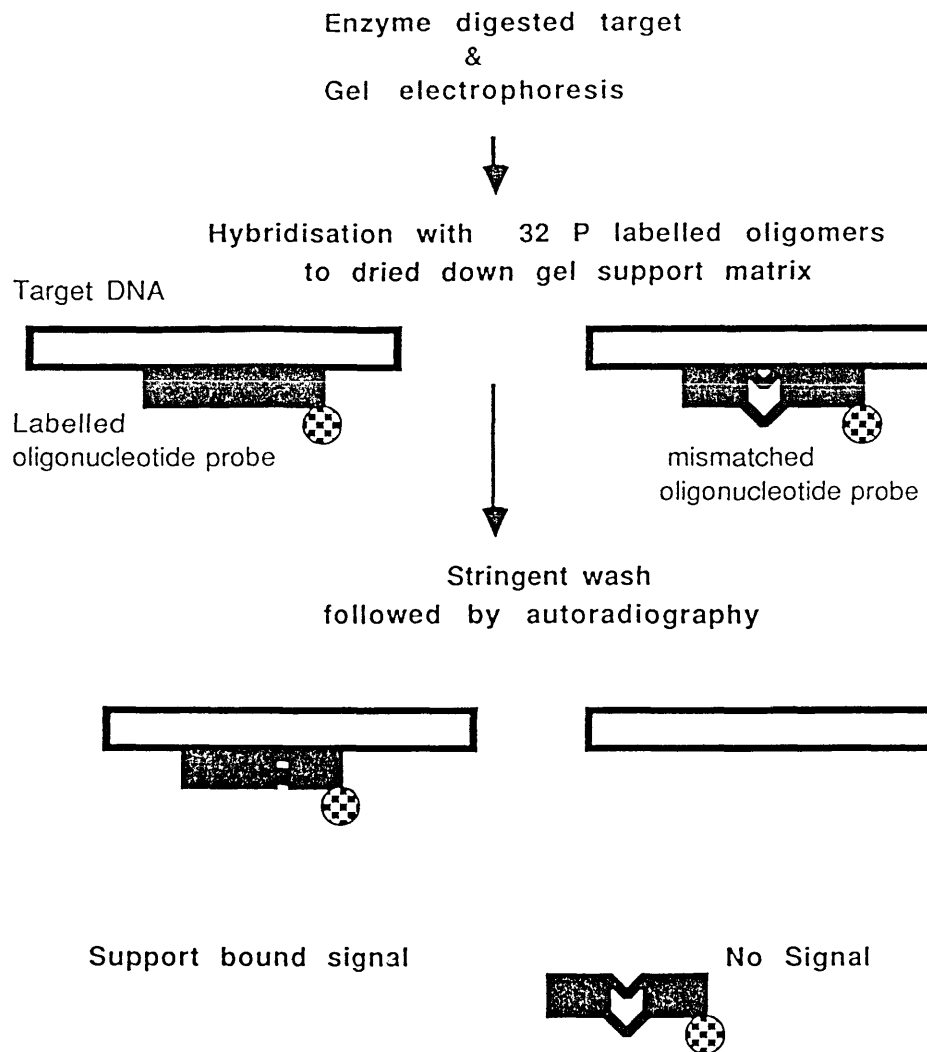


Figure 2 :DETECTION OF SINGLE BASE MUTATIONS USING LABELLED OLIGONUCLEOTIDES

cells. Changes in the ratio of donor to recipient DNA reflected changes in the cell population (Wallace et al., 1986).

1.4 Sandwich hybridisation

The method of sandwich hybridisation was developed by Dunn and Hassel in 1977 as an alternative to the direct filter based hybridisation technique mentioned previously. The aim of this new technique was to eliminate the need for sample pretreatment as well as the application to filter. Moreover, it presented a technique which not only left the specimen DNA free to be used again as a reagent, but also was a method easily carried out, giving clear cut numerical values for interpretation.

The principle of the sandwich hybridisation method is shown in figure 3. In this method an activated solid support is coated with a DNA capture probe. The immobilised probe is used to capture the target DNA (specimen DNA), which is then detected with a second, labelled probe. Both detector and capture probes are designed as two non-overlapping but adjacent sequences complementary to the DNA to be identified. Quantitation of the specific target DNA is achieved by measuring the label bound to the solid support and comparing this with the amount attached in the absence of target DNA.

The method of sandwich hybridisation has been applied to both DNA and RNA samples, and has not been found to be seriously affected by protein or other biological contaminants. Past diagnostic research using this technique has been primarily involved with the detection of microbial pathogens (reviewed by Palva and Ranki, 1985) such as: *E. Coli* and other *Enterobacteriaceae* (Palva, 1983), *Salmonella* (Polsky-Cynkin et al., 1985), Adenovirus in infected cells and nasopharyngeal mucus (Ranki et al., 1983; Virtanen et al., 1983 and Syvanen et al., 1986),

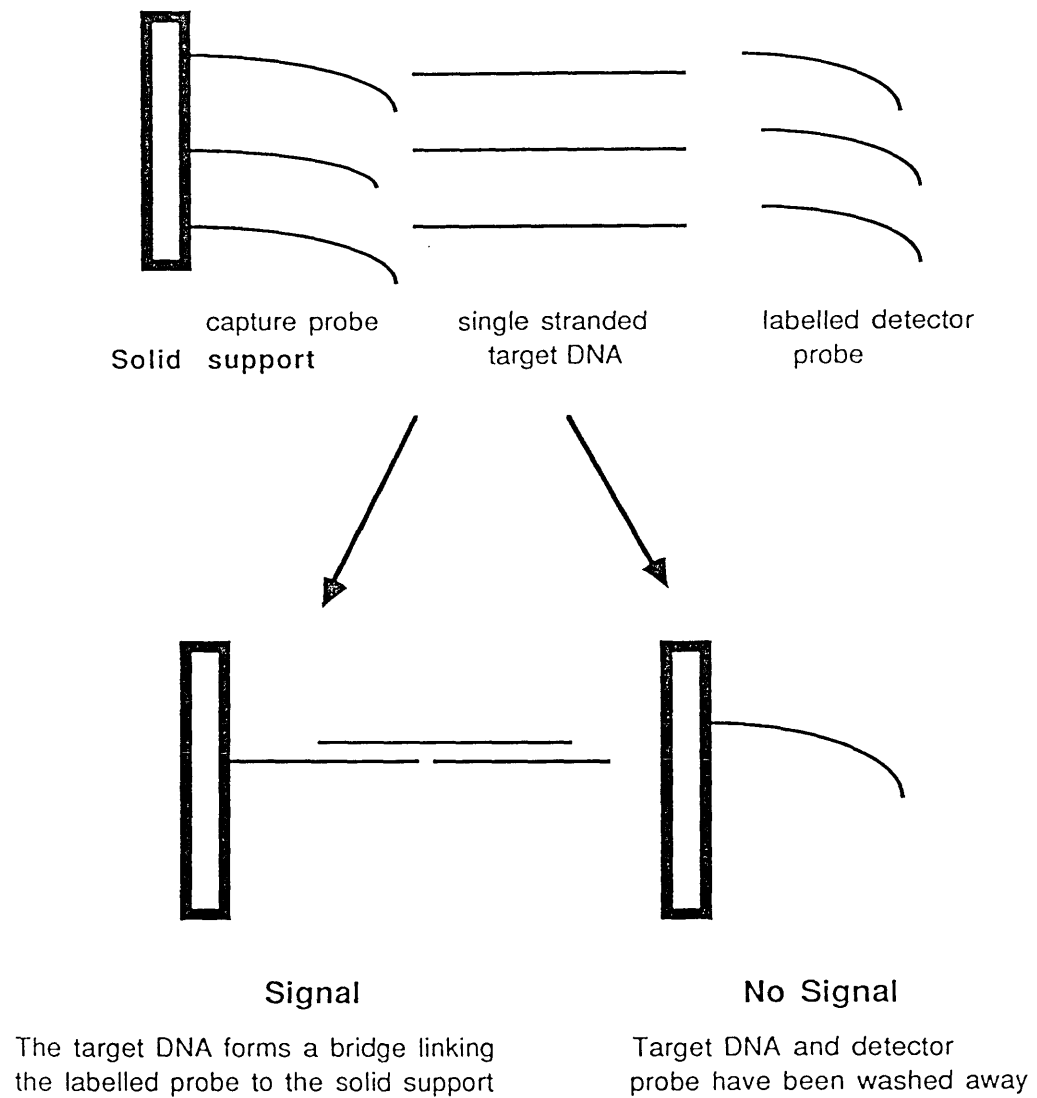


FIGURE 3 PRINCIPLE OF THE SANDWICH ASSAY

Cytomegalovirus in Urine (Virtanen et al.,1984), Chlamydia trachomatis in genitourinary swabs (Palva et al.,1984) and Hepatitis B virus in blood (Urdea et al.,1987).

More recently the same technique has been used to detect Papilloma virus in cervical scrapings (Parkkinen et al.,1986),the presence of which is thought to have an association with cervical cancer (Gissmann et al.,1983).Sandwich hybridisation has also been used in gene expression studies and in understanding the regulation of protein production.As a model for this work,alpha-amylase mRNA from *Bacillus subtilis* was used (Palva et al.,1988).Clearly the quantification of expressed genes will not only be of value to biotechnology,but may also be an important step in the clinical diagnosis of acquired diseases.

Sandwich hybridisation has had less impact on the development of new methods for the qualitative diagnosis of genetically inherited diseases.Application of the sandwich hybridisation strategy to the detection of altered restriction sites in DNA was demonstrated by Langdale and Malcolm (1985).

Using the sickle mutation of the beta-globin gene as target DNA and cloned DNA fragments as capture and detector probes it was shown that all three genotypes could be distinguished.To enable differentiation between the genotypes it was however necessary to enzyme restrict the target DNA with the restriction enzyme Dde I.As the sickle condition is associated with a loss of this restriction site at the site of mutation this genotype is not affected by the restriction enzyme.Detector probe immobilised to the solid support through hybridisation was therefore an indication of the sickle genotype.This strategy,outlined in figure 4.,would however appear to be applicable only to the detection of those mutations where a known restriction site is affected.

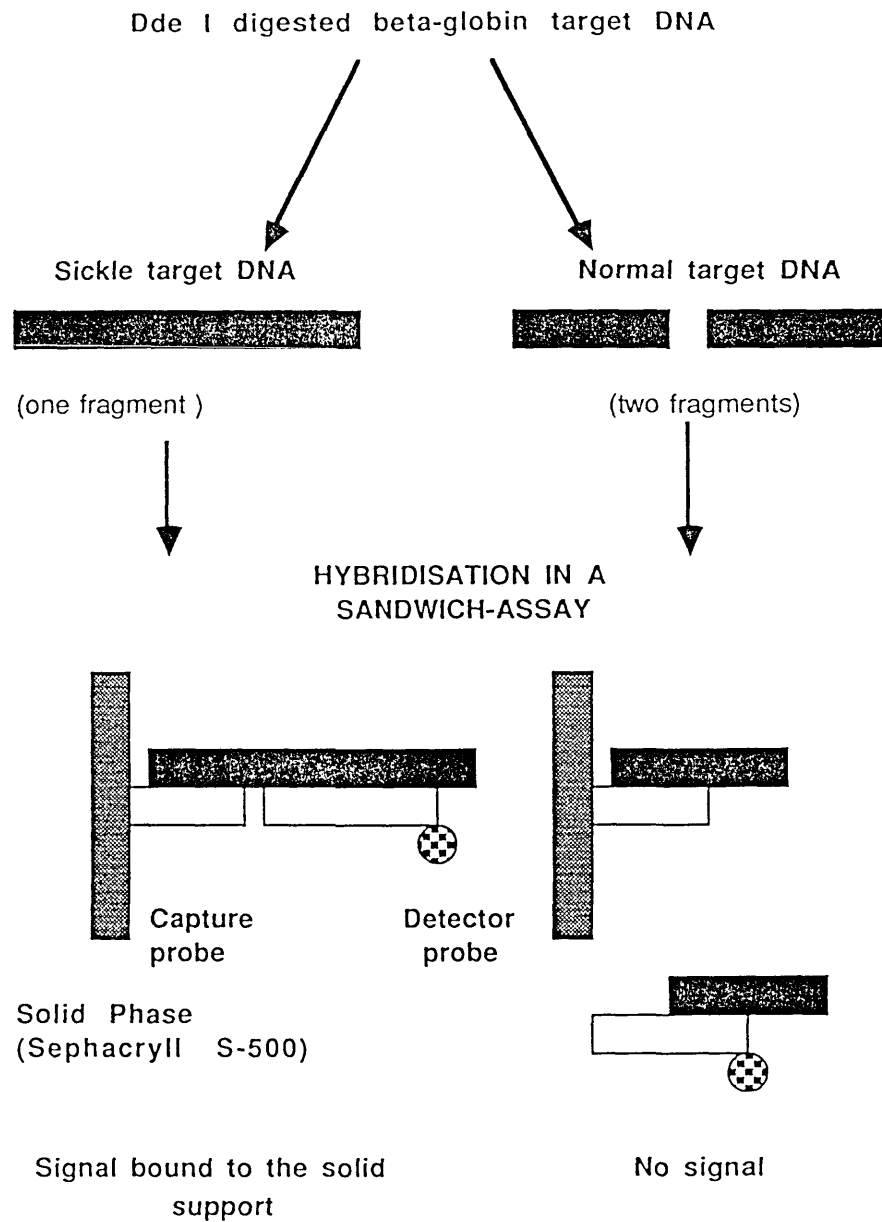


FIGURE 4: DETECTION OF THE SICKLE MUTATION IN THE BETA-
GLOBIN GENE USING THE SANDWICH-ASSAY

(Langdale and Malcolm, 1985)

1.4.1 Solid supports used for sandwich hybridisation
assays

Many support systems and binding methods have been used for the sandwich hybridisation assays. Originally the technique was developed using viral DNA bound to nitrocellulose filters through Southern blotting. Quantitative and irreversible immobilisation was achieved by baking the DNA to the filter at 80°C (Gillespie and Spiegelman, 1965). Binding was thought to occur through hydrogen bonding or a hydrophobic interaction between the DNA bases and the nitrocellulose (Seed, 1982). The complementary target DNA was then hybridised such that the 3' or 5' end protruded as a single-stranded tail, available to further hybridisation by the labelled detector probe. Since then numerous other support systems have been used, some of which are listed in table 2.

Generally DNA immobilisation procedures have influenced the choice of solid support used. Two principle methods have evolved. The first involves immobilisation either through physical or mechanical forces including irradiation and the second uses chemical activation procedures (reviewed by Schott, 1985).

a) **Physical immobilisation**

The methods of physical immobilisation can be subdivided into three groups: adsorption, linkage mediated through ultra violet irradiation and entrapment. Most of these techniques bind the DNA in a non-specific fashion to the solid support, such as cellulose, nitrocellulose, agar, polyacrylamide beads or polyvinyl chloride beads. The mechanism of non-covalent DNA linkage using these

Linkage system	Solid Support	Reference
1.Recombinant DNA		
Non-covalent adsorption	Nitrocellulose filters	Dunn and Hassel (1977)
CNBr	CNBr activated Sepharose 4B	Polsky-Cynkin et al.(1985)
Protein:DNA non-covalent adsorption	Polypropylene	Polsky-Cynkin et al.(1985)
via Carbodiimide activation,also via diazotization	Sephacryls (200,400,500) Sephadex (50) Magnetizable cellulose Cellulose	Langdale and Malcolm (1985)
via reactive epoxide groups	Acrylic beads	Carrico (1985)
Biotin/streptavidine	Streptavidine coated micro-titre plates	Syvanen et al. (1986)
2.Oligonucleotide		
Biotin/avidine	Avidine containing polystyrene beads	Urdea et al. (1987)
Non-covalent adsorption to proteins	Microtitre dish wells	Urdea et al. (1988)

Note:the two categories 1.recombinant DNA and 2.oligonucleotide refer to the type of DNA used in the linkage protocol of the sandwich assay.

TABLE 2 SOME OF THE DIFFERENT SOLID PHASE SUPPORT SYSTEMS USED FOR THE SANDWICH-HYBRIDISATION ASSAY

immobilisation procedures is generally not understood, however a certain level of release from such supports has been shown to occur (Cannon et al., 1985).

Recently a new interest has been expressed in the methods involving ultraviolet irradiation. Although the exact mechanism is unknown UV irradiation appears to crosslink the DNA to insoluble supports in a stable fashion, which is strong to desorbing conditions and elevated temperatures. Using ultraviolet irradiation recombinant DNA probes have been immobilised to microtitre plates (Nagata et al., 1985) and to fibreglass (Sheldon et al., 1972). Improvements to this method have involved the U.V. crosslinking of DNA specific binding proteins, which bind at greater efficiency and stability (Shouten, 1985), than DNA itself.

With respect to immobilisation using chemical activation, methods have been designed so that DNA can either be immobilised specifically to the 3' hydroxyl or 5' phosphate of the sugar backbone or in a random fashion via the amino groups on the bases.

b) Chemical immobilisation

One of the first methods to be introduced was carbodiimide mediated attachment via the 5' phosphate group. This was originally achieved by condensation of synthetic polynucleotides with cellulose in an anhydrous solution (Gilham, 1964). As this method could only be applied to polynucleotides prepared chemically in anhydrous solutions, a more general method was developed, permitting activation in aqueous solutions (Gilham, 1968). The reaction mechanism in both is one of covalent attachment through the formation of phosphodiester bonds

between the 5' phosphate and the hydroxyl group on the solid support (Naylor and Gilham, 1966). The structure of carbodiimide is illustrated in figure 5.

Immobilisation efficiencies have been reported to vary with DNA size ranging from 60-70% for mononucleotides to just 20-30% using polynucleotides such as polyadenylic acid (Gilham, 1968) and to similar percentages using longer DNA fragments. Work by Astell and Smith (1972) suggested that improvements in these coupling yields could be achieved by increasing the concentrations of carbodiimide present in the reactions.

Modifications of the coupling procedure using carbodiimide have included the introduction of carboxyl groups to the ends of single-stranded DNA by treatment with 4-diazobenzoic acid. The carboxyl groups are then linked to aminopentane-Sepharose using water soluble carbodiimide (Dickerman et al., 1978). Other investigations have adapted the technique for the immobilisation of single and double stranded DNA (Rickwood, 1972; Allfrey and Innoue, 1978) as well as the immobilisation of RNA (Metz and Brown, 1969). The main support systems used have been polysaccharide supports such as Sephadexes and Sepharoses and celluloses (Shih, 1974).

Methods commonly used for covalent immobilisation of single stranded recombinant DNA by random base linkage include: coupling by diazotization (Noyes and Stark, 1975), condensation with cyanogen bromide (Poonian et al., 1971) and coupling via bisoxirane activation (Moss et al., 1981).

Coupling by diazotization is one of the few methods of random base immobilisation that has been adapted for use in sandwich hybridisation assays (Langdale and Malcolm, 1985). The procedure consists of the covalent attachment of an aromatic amino compound to the solid support, in the first step. In the second, the amino function is

1 - cyclohexyl - 3 - (2 - morpholinoethyl) carbodiimide
metho - p - toluene sulfonate

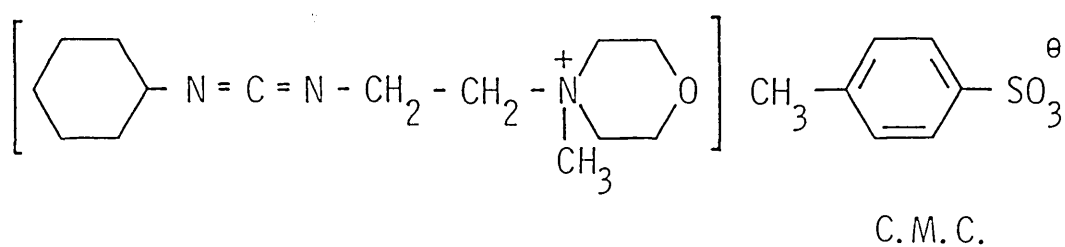


FIGURE 5 THE CHEMICAL STRUCTURE OF CARBODIIMIDE
(water soluble)

diazotized and is coupled with denatured DNA.

Generally the procedures for each of these steps follow the protocols of Noyes and Stark (1975) and Seed (1982) respectively. Two independent coupling groups were generated for this type of immobilisation, the first was diazobenzylloxymethyl (DBM) cellulose (1975) and the second, diazophenylthioether (DPTE) cellulose (1982). However, the availability of DPTE cellulose bound DNA to hybridise was greater (Bunemann et al., 1982 b)) and therefore more commonly used. The reported yields have varied with the solid supports used, so that for Sephadex G-25 the binding efficiencies have been between 13-31% (for sheared recombinant DNA) and for Sephacryl S-1000 the yields have been between 63-80% (Bunemann et al., 1982 b)) of the initial input DNA concentration.

Condensation reactions with CNBr-activated polysaccharides can efficiently immobilise DNA, RNA and polyribonucleotides via their base amino groups (Arndt-Jovin et al., 1975). Activation of the resins with CNBr forms a reactive imidocarbonate which acts to couple DNA at mildly alkaline pH. Immobilisation yields have been reported to be unaffected by DNA size in the range of 0.5-5 kb and vary, depending on the solid support matrix used. The most efficient supports are epichloro-hydrin- desulfated agarose which resulted in RNA immobilisation yields of 50-70%, and sepharose which resulted in yields of 90% with DNA (Siddell, 1978).

Finally, bisoxirane activation has been used to link DNA to cellulose with an efficiency of 90% binding for recombinant fragments of approximately 500 base pairs in length. The technique utilises a bifunctional oxirane, 1,4-butanediol diglycidyl ether, which links the DNA to the cellulose or agarose matrix by coupling to the secondary or tertiary amines on the DNA and the hydroxyl groups of the support system. No effect of DNA size has been observed in the range of

50-600bp (Lazo,1984) and 90% of the immobilised DNA has been reported to be available to RNA hybridisation (Moss et al.,1981,Biagioni et al.,1978).The immobilised DNA matrix generated by this method has primarily been used for affinity chromatography.

Recently a method has been reported,for the 5' end immobilisation of single-stranded recombinant DNA fragments,which specifically uses poly (dT) cellulose as a support matrix and enzymic linkage reactions (Goldkorn and Prockop,1986).The technique also uses complementary (dA) tailed DNA fragments to assist as linkers to the enzymic ligation between the poly (dT) cellulose and the DNA to be immobilised.Yields of up to 45% of the original input DNA were successfully immobilised using this method,of which 35-41% of the immobilised DNAs were available to hybridisation.

1.4.2 The kinetics and sensitivity of sandwich hybridisations using recombinant DNA fragments

The principle parameters affecting the kinetics of hybridisation in a three component sandwich hybridisation assay are considerably more complex than the two component hybridisations (occurring either as a one-phase reaction in solution,or as a two-phase reaction onto a filter),reviewed by Meinkoth and Wahl (1984).During the sandwich assay,at least five different annealing reactions occur one of which involves the self-annealing of the denatured target DNA.Furthermore,the kinetic behaviour is affected by the concentration of free target DNA used in a sandwich assay,which is generally not used in excess of the other nucleic acid constituents.

The conditions found to suit efficient hybridisation in a sandwich assay have included the use of excess detector probe.An increase in

target detection has been reported to occur with increasing concentrations of labelled detector probe (Ranki et al.,1983). One report described a further increase in target detection,over a three to four hour incubation,with the addition of dextran sulfate (Polsky-Cynkin et al.,1985).No difference in hybridisation efficiency was found after a 17 hour incubation period (Ranki et al.,1983).

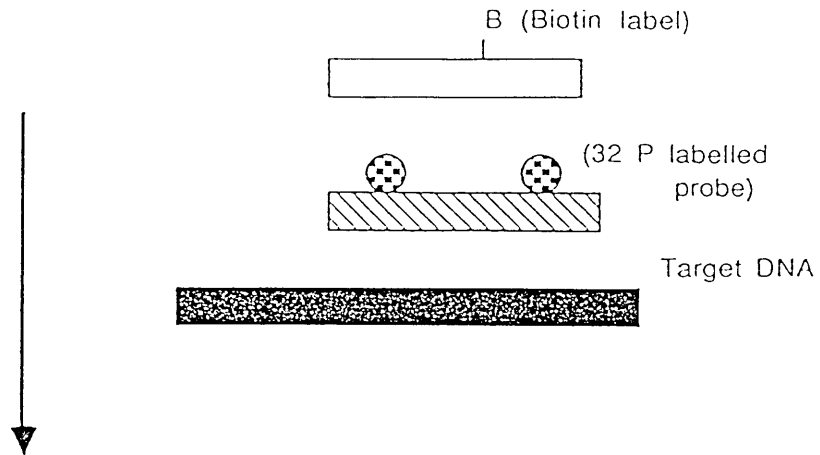
To maximise the sensitivity of the sandwich assay an excess of capture probe was also required,although this was associated with a respective increase in background binding (Ranki et al.,1983). Due to the nature of the sandwich-assay even small differences in signal can be measured over background,either by Cerenkov counting when radioactively labelled probes are used (review,Berger and Krug,1985), colorimetrically or through the emission of light,when non-radioactive labelling techniques are used.A five fold increase in detection has been recorded over the limits found using filter hybridisations (Ranki et al.,1983).

Most investigations into the detection limits of the sandwich assay,assumed a two-fold difference in signal over background to be a positive signal.Alternatively the limit of positivity was determined from the negative controls,by using a statistical cut-off line at 95 per cent of the negative distribution. Using this criterion the limits of target detection in a sandwich-assay,using recombinant DNA probes,have been reported to be between 50 (Ranki et al.1983) and 5 attomoles (Langdale and Malcolm,1985).

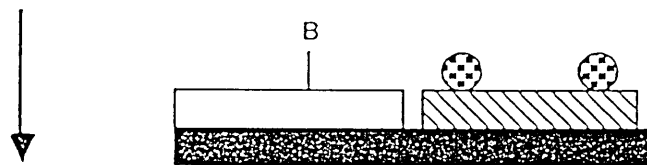
1.4.3 Solution sandwich hybridisation strategies

As solution hybridisation is inherently faster than solid phase hybridisation many adaptations of the original sandwich hybridisation assay have been designed.One method (illustrated in figure 6.) has

THREE HYBRIDISATION COMPONENTS



HYBRIDISATION IN SOLUTION



COLLECTION OF HYBRIDS ON A SOLID SUPPORT

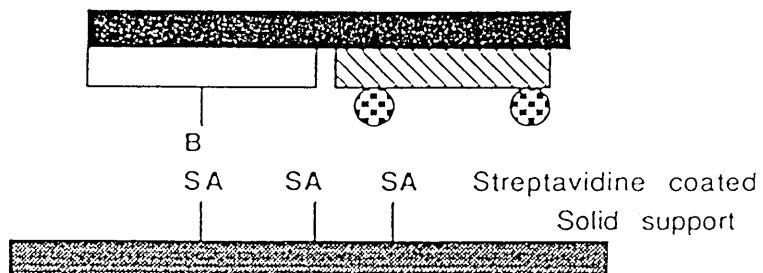
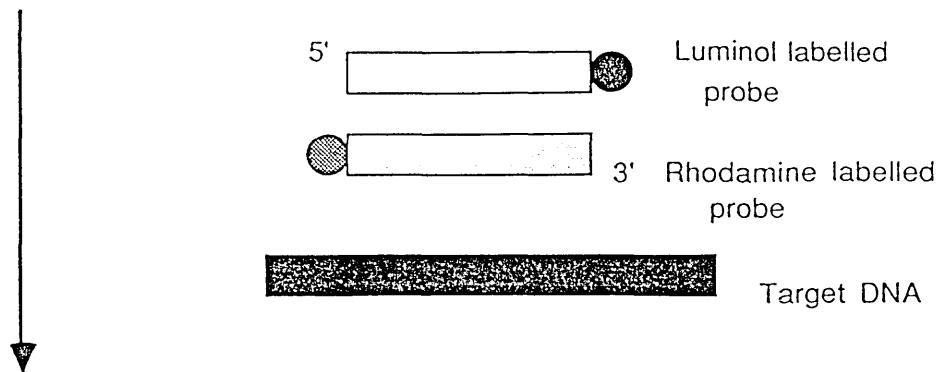


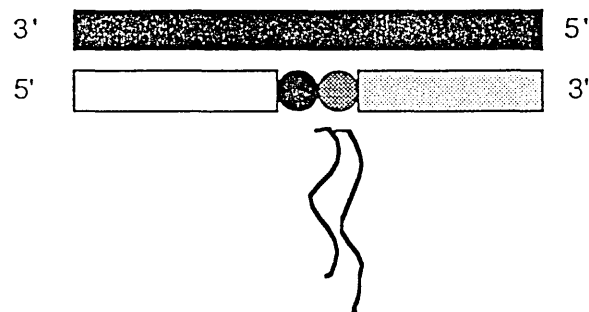
FIGURE 6: DIAGRAMATIC REPRESENTATION OF A SOLUTION HYBRIDISATION ASSAY

(As described by Syvanen et al. 1986)

THREE HYBRIDISATION COMPONENTS



HYBRIDISATION IN SOLUTION



ENERGY TRANSFER RESULTING
IN FLUORESCENCE

FIGURE 7 :A HOMOGENEOUS ASSAY SYSTEM BASED ON
AN ENERGY TRANSFER REACTION

(Heller and Morrison,1985)

been to capture the hybrid onto the solid support after the hybrid complexes have formed. By labelling the capture probe with a ligand such as biotin the hybrid complexes can be separated from the incomplete hybridisations by binding to streptavidin-coated solid supports (Syvanen et al. 1986).

Another strategy for a homogeneous assay (illustrated in figure 7.) involves the use of two labelled probes, which, when in close proximity, give an energy transfer, resulting in light emission. Heller and Morrison (1985), for example, labelled the 3' end of one probe with a chemiluminescent fluorescent donor (e.g. luminol) and the 5' end of the other with an absorber/ emitter (e.g. rhodamine). Upon hybridisation of both to the target DNA, energy transfer occurred which lead to quantifiable light emission. Other labels have also included immunological labels such as glucose oxidase and horseradish peroxidase-labelled anti-RNA:DNA hybrid antibodies. When hybridised to the target DNA these labels resulted in an energy transfer permitting colorimetric detection (reviewed by Matthews and Kricka, 1988).

1.5. Oligonucleotide immobilisation

Prior to the advent of molecular cloning and nucleic acid blotting techniques, oligonucleotides were most commonly used as primers for DNA synthesis (Wu, 1972, Panet and Khorana, 1974) or as affinity reagents to purify specific mRNAs by oligonucleotide-cellulose chromatography (Aviv and Leder, 1972). The oligonucleotides synthesised were generally short mononucleotide sequences and immobilisation procedures were usually performed using 5' covalent end-linkage by carbodiimide activation (Gilham, 1964) to cellulose support matrices.

Later, as oligonucleotides were more rapidly and easily produced (Gait, 1984), their sequence specificity upon hybridisation was used to

isolate cloned DNA sequences, to identify messenger RNA sequences and to detect unique genes for diagnosis (reviews, Reyes and Wallace, 1983; Itakura et al., 1984). The sequence specificity of oligonucleotides also lead to the development of techniques such as DNA sequencing, site-directed mutagenesis and in situ hybridisation. For all of these manipulations oligonucleotides were primarily used as free hybridising detector probes and rarely immobilised with the exception of the oligonucleotides immobilised for affinity chromatography.

Apart from the well investigated approach of covalently linking mononucleotides using carbodiimide, alternative 5' end coupling strategies were established. One involved the attachment of 5'-NH-terminated oligo (dT) to Sepharose by coupling with CNBr activated cellulose. Immobilisation occurred in high yields exclusively binding the oligonucleotide by the 5' terminus. Activated cellulose support matrices were also reported to bind oligonucleotides by the same functional group.

As an alternative insoluble support matrix, cross-linked poly (vinyl-alcohol) gels were used. Oligomers of four or more monomeric units were immobilised via ester linkage with their 5'-phosphate groups to the free hydroxyl groups of the support. Similar immobilisation procedures could also be carried out on soluble poly (vinyl alcohol) which could then be irreversibly adsorbed as polyanion on the anion exchanger DEAE-cellulose (Schott, 1985).

Most oligonucleotide based adsorbents used in affinity chromatography today and manufactured by industry still rely on the same coupling strategies. The solid support matrices used are primarily agarose and cellulose derived support systems, although Sephadexes (a bead form, cross-linked dextran gel) have also been used (Coffin, 1974).

Oligo (dT) cellulose is prepared by covalent coupling using a modification of the original carbodiimide linkage procedure

(Lungforetagen, 1979). Poly (U or A) Sepharose is generated using the cyanogen bromide method of linkage. Immobilisation is however achieved by multiple point covalent attachments (Bywater, 1974, referred to by Lungforetagen, 1979) via the tautomeric enolate form of the nucleotide, to ensure greater stability.

Linkage via the 3' hydroxyl group has not been frequently used for the immobilisation of oligonucleotides. One report however made use of 3' end immobilisation using water soluble carbodiimide, in order to generate a template system for DNA replication, using poly (dT) with an average chain length of 80 nucleotides (Panet and Khorana, 1974). Other techniques have now arisen as a result of the advances made in oligonucleotide synthesis (Gait, 1984).

One of the new linkage systems for solid phase synthesis of oligonucleotides, which has been of particular interest, is linkage via urethane moieties (Sproat and Brown, 1985). An aryl diisocyanate is used to attach an appropriately protected 2' -deoxyribonucleoside bearing a free hydroxyl group to the amino group of a solid support, for example long chain alkylamine controlled pore glass.

Urethane linkage was shown not only to be less susceptible to unwanted chemical cleavage than the ester bond, it also could be used for the immobilisation of any of the four nucleosides. Conventional succinate (figure 8.) linkage, on the other hand, required the preparation of four protected 2'-deoxyribonucleoside-3'-O-succinates, which then required conversion to active esters prior to immobilisation. Immobilisation using the "urethane chemistry" might present an alternative method of the future for the 3' end-linkage of oligonucleotides with high coupling efficiencies to solid supports.

More recently a teflon fibre support has been developed as a substrate for oligonucleotide synthesis using phosphite-triester

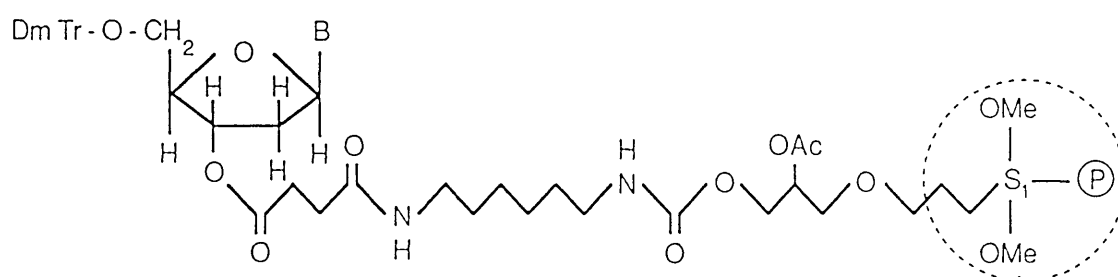


FIGURE 8 Deoxyribonucleoside linked to Long Chain Alkylamine
Controlled Pore Glass

chemistry (Lohrmann et al., 1984). Using this support system oligonucleotides were synthesised which could later be used for the affinity chromatography of sequence specific binding proteins (Duncan and Cavalier, 1988). Perhaps this approach will be one of the most efficient ways of coupling oligonucleotides.

In the last four years one of the main focuses in research has been the possibility of using short oligonucleotide probes immobilised on to solid supports as tools in the detection of pathogenic agents or genetically aberrant conditions. This interest has been prompted by the rapid advances made in the technology of oligonucleotide synthesis and purification.

Oligonucleotides have been immobilised to a variety of microgranular support systems using the chemistries already available from the advances made in affinity chromatography and the strategies developed for the coupling of recombinant DNAs. These support matrices have been used in preference to the more traditional filter support systems for a number of reasons. Firstly, no ideal filter support systems are available which can irreversibly bind oligonucleotides without a significant loss of material from the matrix. Secondly, the hybridisation properties of oligonucleotides have already been characterised for microporous supports, as a result of work carried out in the development of affinity chromatography.

It is thought that by using oligonucleotides bound to microgranular support systems one is more likely to generate conditions approximating those of solution, where the rates of hybridisation are 10-100 times faster (Bunemann, 1982 b)).

Oligonucleotides immobilised by random linkage would probably display a much smaller change in rates than those attached through end-linkage. Hybridisation rates are less likely to become diffusion

Linkage system	Solid support	Reference
<u>I) 5' end linkage</u>		
Carbodiimide	Magnetic beads (hydroxyl, carboxyl, amino)	Lund et al.(1988)
Carbodiimide	Submicron-latex particles (hydrazide functionalised particles)	Wolf et al.(1987)
Carbodiimide	Sephacryl (S-500), Controlled pore glass (amine), polystyrene (amine)	Ghosh and Musso (1987)
Carbodiimide	Whatman 3mm paper or powder	Gilham (1971)
<u>II) 3' end linkage</u>		
Urethane linkage	Controlled pore glass (amine)	Sproat and Brown (1985)
Phosphotriester linkage	Teflon fibre	Duncan and Cavalier (1988)
Carbodiimide	Cellulose Cellex (powder)	Panet and Khorana (1974)
<u>III Random covalent linkage</u>		
via diazotization	Magnetic beads (hydroxyl)	Lund et al.(1988)
via CNBr	CNBr activated magnetic beads	Lund et al.(1988)
Biotin/avidine	Avidine polystyrene	Urdea et al. (1987)
CNBr	Sephacryl (S-500)	Ghosh and Musso (1987)
CNBr	Sepharose	Bywater et al. (1974)
	Sephadex	Coffin et al. (1974)

TABLE 3 SOLID PHASE SUPPORT MATRICES USED FOR
COVALENT OLIGONUCLEOTIDE IMMOBILISATION

limited as the reactions can be performed in small volumes and can be shaken with greater ease. Simple shaking of filter supports has been shown to increase the rates of hybridisation (Flavell et al., 1974). Furthermore, many microgranular support systems and the chemistries used to immobilise DNAs to them, are thought to generate less steric interference than the filter support systems and provide greater accessibility of the attached DNAs to hybridisation. Some of the microgranular support systems and chemistries used for oligonucleotide immobilisation are listed in table 3.

This new approach to hybridisation has been used primarily on an experimental basis and has not been applied to the detection of specific DNAs, with the exception of work done by Urdea et al. (1987).

Using a modified procedure of the sandwich hybridisation assay (Hansen, 1985) a novel method for the rapid detection of hepatitis B virus in human serum was developed. The target DNA is captured in solution using a set of twelve sequence specific oligonucleotide probes (type A) each with a constant region of 20 bases complementary to the solid phase oligonucleotide capture probe (type C). The type C probes are biotin labelled and can immobilise target: type A complexes by reacting with an avidin containing polystyrene bead. Other oligonucleotides also complementary to the target DNA are used as sequences complementary to an oligonucleotide amplification multimer, which in turn hybridises to oligonucleotides bearing a horseradish peroxidase label. Detection is achieved with either a colorimetric or chemiluminescent output substrate for the enzyme. The strategy, illustrated in figure 9., is sufficiently sensitive to permit single gene detection in 5 μ g of mammalian DNA in 4h.

A novel method for the rapid detection of specific nucleotide sequences in crude biological samples without blotting or radioactivity

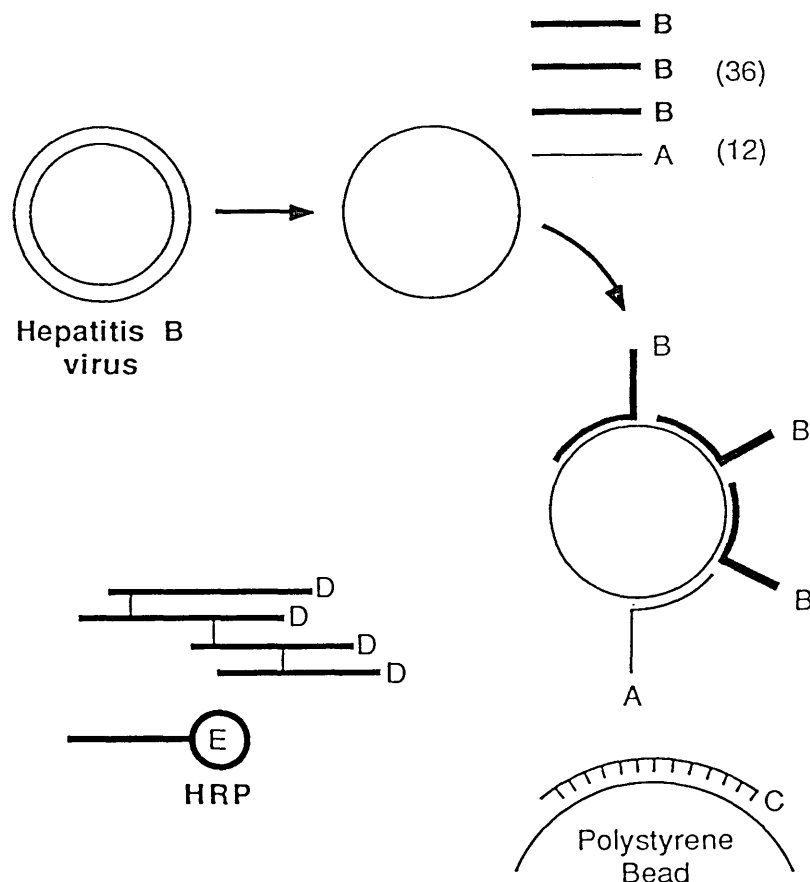


FIGURE 9

(URDEA ET AL.,1987)

Five types of synthetic oligodeoxyribonucleotides were employed : type A is comprised of a set of twelve single stranded capture probes,where one varying portion ,30 nucleotides in length is complementary to a specific sequence of the HBV genome and a second constant region of 20 nucleotides is complementary to the solid phase capture probe ,type C.Type B is a set of 36 single stranded labelling probes,where one portion (30 nt.) is complementary to a specific sequence of the HBV genome and a second constant region (20nt.) is complementary to the amplification multimer ,type D.Each multimer is made up of chemically cross-linked oligodeoxyribonucleotides.Type C is 5' end-labelled with a biotin moiety and is bound to avidine containing polystyrene beads.Type E is an 18 nt. 5' HRP labelled probe.Hybridisation reactions with all five types of probe and HBV target DNA,results in the immobilisation of HRP to the solid support,which can be detected through colourimetric detection procedures.

1.6 Oligonucleotide hybridisation

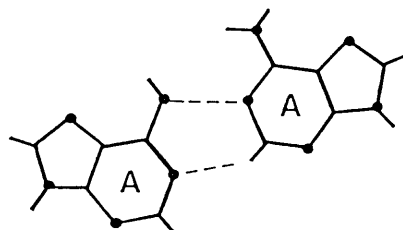
Oligonucleotides (between 14-20 bases) used as labelled probes in the hybridisation to immobilised nucleic acids show decreased hybrid stability in comparison to longer probes (over 50 bases). An empirical formula has been determined to estimate the optimal conditions for their hybridisation (Wallace et al., 1979). The temperature at which 50% of the duplexes dissociate (T_d) when hybridisation is performed under standard conditions (0.9M NaCl) is:

$$T_d (^{\circ}\text{C}) = 4(\text{G} + \text{C}) + 2(\text{A} + \text{T})$$

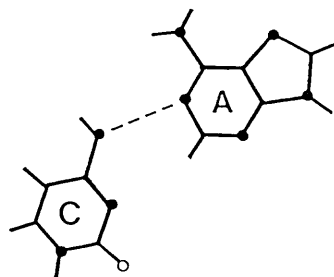
The higher the GC content of a duplex the greater the duplex stability. It has generally been found that by setting the hybridisation temperature 5-10 $^{\circ}\text{C}$ below the melting temperature of the oligonucleotide one can achieve differentiation between exactly matched and mismatched hybrid sequences (Conner et al., 1983).

The stability of duplexes formed between mismatched strands is decreased according to the number base mismatches and the position of the mismatch relative to the end of the duplex. For oligonucleotides shorter than 20 bases every mismatched base pair is thought to decrease the melting temperature by approximately 5 $^{\circ}\text{C}$ (Wallace, 1983). The exact thermodynamic stability of mismatched bases is, however, thought to depend on the nature of the mismatch (Chuprina and Potev, 1985) as well as the hybrid stability of the neighboring bases (Martin, 1985; Arnold et al., 1987; Freier et al., 1985). At room temperature the most stable mismatches are those containing guanine and the least stable are those containing cytosine. At higher temperatures pyrimidine-pyrimidine mismatches become least stable (Martin, 1985). The proposed interaction of mismatched base pairs is

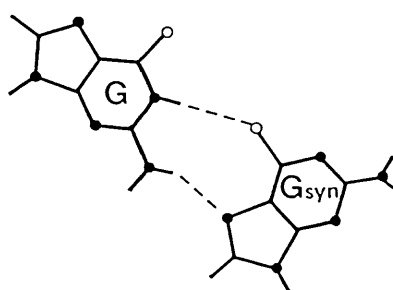
Purine : Purine
2 2



Purine : Pyrimidine
2 3



Purine : Purine
3 3



Pyrimidine: Pyrimidine
3 3

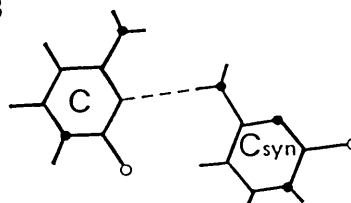


FIGURE 10 Interaction of mismatched basepairs
(Chuprina and Poltev,1985)

illustrated in figure 10.

For hybridisation conditions under which duplexes with one or more mismatches will not form, Suggs et al (1981) have suggested a hybridisation temperature of 2-5°C below the calculated T_d in 1 M Na⁺. Another method of achieving differential hybridisation has been through adjusting the stringency of the post-hybridisation washes (Thein and Wallace, 1986). However, the conditions for eliminating mismatch hybridisations must be determined empirically for each oligonucleotide. Using this strategy oligonucleotides have been used as labeled probes to detect single base changes in a number of unique genes (see table 4.).

A technique which uses tetramethylammonium chloride to eliminate the dependence of T_d on the G C content of the probe was described by Melchior and von Hippel (1973). Tetramethylammonium chloride binds to AT-rich DNA polymers, abolishing the preferential melting of AT versus GC base pairs. Thus the hybridisation of these probes to genomic DNA or to recombinant DNA libraries (Jacobs et al., 1988) is a function of probe length and not base composition. This reagent has been reported to be particularly suited for detecting and distinguishing between normal and mutant alleles in the human genome (Kidd et al, 1983), since mismatches between an oligonucleotide probe and complementary DNA sequence are still sufficient to destabilise the duplex structure of the hybrid (DiLella et al, 1981).

The substitution of bases by analogues also modifies thermal stability, double helical conformation or base-pairing pattern, resulting in difference in hybridisation strength selectivity. Analogues have been found that can decrease duplex stability both when in a position of duplex complementarity and in a position of mismatch (Millican et al., 1984). Others have been found which stabilise the duplex irrespective of base composition (Habener et al., 1988). Recently

1. Beta S Hgb mutations		
A		wild type
CTCCTG	GGAGAAGTCTGC	
T		sickle
G		wild type
CTCCT	AGGAGAAGTCTGC	
A		c
2. Alpha-1-antitrypsin		
G		wild type
ACCATCGAC	AGAAAGGAC	
A		Z
A		wild type
CAGCACCTGG	AAAGAACTC	
T		S
3. Phenylketonuria IVS12		
G		wild type
TCCATTAACA	TAAGTAATTT	
A		IVS12
4. NRas oncogene (N12)		
G		wild type
GGA	CAGGTGGTGTGGGAA	
N		N12pl.
		(N = A,C,T)

TABLE 4 SOME OF THE SINGLE BASE CHANGES THAT
HAVE BEEN DETECTED USING OLIGONUCLEOTIDES

The anti-sense strand sequences for normal and mutant oligonucleotides, used to identify the disease mutations are shown. The single base changes associated with the disease are shown below the probe sequence.

a third type of analogue (illustrated in figure 11.) has been developed which demonstrates increased hybrid stability, when in a position of sequence complementarity, and decreased hybrid strength when present in a mismatched duplex (Chollet et al., 1986, 1988). The different structural classes of oligonucleotide analogues are reviewed by Zon (1988).

The rate of helix formation for matched duplexes has been suggested to be the same as for single base pair mismatched duplexes (Wallace et al., 1979). Differential hybridisation between the duplexes must therefore be the result of differences in the rate of dissociation. As a consequence of this the time used in the post-hybridisation wash is critical.

The rate of renaturation of DNA in high salt was found by Wetmur and Davidson (1968) to be proportional to the square root of the length of the reacting single strands (Lee and Wetmur, 1972). Similarly the rate of renaturation is affected by temperature and the concentration as well as complexity (number of nucleotides in non-repeating sequence for hybridisations done in 1 M Na⁺) of the oligonucleotide probe (Crothers et al., 1965). The time ($t_{1/2}$) required for half the probe to anneal with the immobilised DNA sequence is:

$$t_{1/2} = \frac{\ln 2}{kC}$$

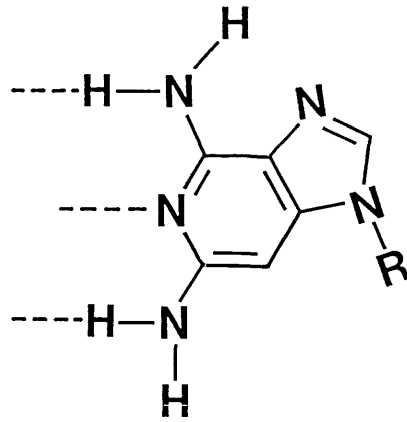
Where k = first-order rate constant

(litres mol/ nucleotide/sec)

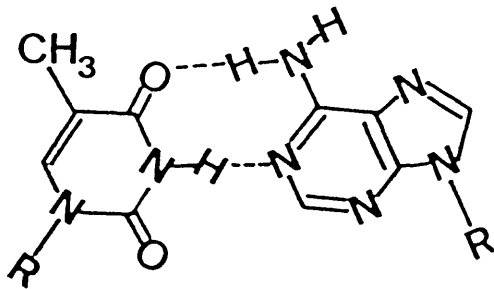
C = probe concentration (mol nucleotide/litre)

L = length of the probe in nucleotides

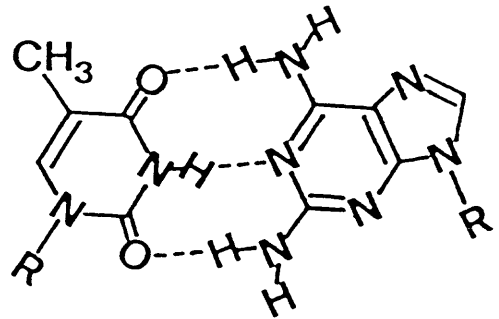
N = complexity of the probe



Base analogue 2-aminoadenine
(2,6-diaminopurine, D)



T : A



T : D

FIGURE 11 THE BASE ANALOGUE 2-AMINOADENINE

(Chollet et al.,1986)

Substitution of adenine by 2-aminoadenine 1 (2,6-diaminopurine) increases the stability of the Watson and Crick base pair with thymidine by an additional hydrogen bond between the 2-amino group on the purine ring and the 2-oxo group on the pyrimidine ring.

The effects of length and GC content are not only critical to the stability of the hybrid complex (review, Smith, 1983) and renaturation rates, they also determine the probes sequence specificity. To be unique an oligonucleotide probe must contain at least 'N' nucleotides where:

$$4^N > 2 \times \text{no. of base pairs in the target genome}$$

As the haploid human genome contains 3×10^9 bases, giving N a value of 17, an oligonucleotide must have a minimum length of 17 bases before it can be used as a specific hybridisation probe for the human genome (Thomas, 1966). The greater the GC content of the probe, the more likely it is to detect unique sequences of the human genome, as repetitive sequences tend to be AT rich.

1.7 Oligonucleotide detection

Most of the labelling strategies developed for the labelling of long recombinant fragments have also been used on oligonucleotides. Two main approaches have evolved for the labelling of DNA. One is direct labelling, in which a label is attached directly via a covalent bond. Examples of labels used for oligonucleotide probe assays are listed in table 5. The second is indirect labelling, in which either a hapten is attached to the DNA and detected by using a labelled binding protein with specificity for the hapten or a hybrid formation is detected by using a binding protein with specificity for long double stranded DNA.

Oligonucleotides labelled directly with radioisotopes are most commonly labelled with a single ^{32}P isotope which is covalently attached to the probe using the enzyme T4 kinase. Their use as highly

I.Direct labels for probes		Reference
Phosphorus-32		Studencki and Wallace (1984)
Sulfur-35	:	Collins and Hunsacker (1985)
Poly AMP		
Fluorescein		Smith et al.(1985)
Alkaline Phosphatase		Seriwatana et al. (1987)
Horseradish peroxidase		Urdea et al.(1987)
Rhodamine		Urdea et al.(1988)
Texas Red, Isoluminol		
II.Indirect labels for probes		
Primary label	Secondary label	Reference
Biotin	Streptavidine/ alkaline phosphatase	Alves et al.(1988)
	Anti-biotin hybridoma peroxidase	Agrawal et al.(1986)
Fluorescein (isothio- cyanate)	Anti-fluorescein and an alkaline phosphatase labelled 2ndary antibody	Kumar et al.(1988)

TABLE 5 OLIGONUCLEOTIDE LABELLING STRATEGIES

specific probes for single copy gene detection in the human genome has however been hampered, in part, by the long exposure times required (Studencki and Wallace, 1984). One way of increasing the speed of detection has been by increasing the specific activity of the probe, in other words the amount of bound label. Another approach has been to amplify or enrich the target sequence to be analysed.

Oligolabelling improves the specific activity of the probe, but the overall increase in signal is limited by the number of isotopes added. Recently Studencki and Wallace (1984) developed a primer extension method of increasing the specific activity of oligonucleotide probes 10-20 fold.

Another strategy has been to use the enzyme terminal deoxynucleotidyl transferase to add labelled dAMP residue to the 3' end of oligonucleotide probes (Collins and Hunsaker, 1985; Schott and Schrade, 1984). The sensitivity of these probes was reported to be 30 fold greater than 5'-end-labelled probes and was comparable to that of a nick-translated plasmid probe of high specific activity. Thermal stability and sequence specificity were not affected by controlled tailing (up to 30 nucleotide additions) of the oligonucleotide (Woodhead personal communications).

Increasing specific activity of the probe can also be achieved through the construction of "amplification multimers". One reported example of such a multimer was in a non-isotopic assay where the multimer was used as an indirect labelling system (Urdea et al., 1987). Oligonucleotides were synthesised with a N4-alkylamino deoxycytidine derivative and later cross-linked with a bifunctional cross-linking agent. Each oligonucleotide was designed such that it was complementary to an overhanging sequence of the probe hybridising directly to the target DNA. Using the multimer it was possible to amplify the detection limits 50-100 fold.

Apart from ^{32}P few of the other radioisotopes have been used for oligonucleotide labelling, apart from ^{35}S , which has recently been used to prepare an oligonucleotide probe with a sensitivity comparable to that of a $5'$ ^{32}P end-labelled probe (Collins and Hunsaker, 1985). The advantages of using ^{35}S were reported to be increased isotope stability and a higher resolution upon autoradiography. It was suggested by Collins and Hunsaker, that another advantage might be increased speed of detection (of up to 100 fold) if an imaging photon detector system, recently developed by Surface Science Laboratories (Palo Alto Calif.) was used.

PCR amplification or enrichment of the target sequence has recently become a new approach to solving the problem of probe sensitivity. By selectively increasing the concentration of specific target prior to hybridisation with an oligonucleotide probe, one not only eliminates the need for high specific activity labelling, but also a significant amount of background hybridisation. Even under stringent conditions oligonucleotides tend to create substantial background hybridisation, often obscuring the bands or signals of interest.

Saiki et al. (1985, 1988) developed a technique of gene amplification by a polymerase chain reaction (PCR). The technique (illustrated in figure 12.) requires specific oligonucleotide primers (20mers or longer) for each strand of template target DNA, which are extended using the Klenow fragment of DNA polymerase I. Through repeated rounds of denaturation, reannealing and synthesis, the target sequence can be amplified several thousand fold, to concentrations which can easily be detected by gel electrophoresis.

A heat stable DNA polymerase enzyme, derived from the microorganism Thermus aquaticus, known as Taq polymerase (Chien et al., 1976; Tindall and Kunkel, 1988), has recently enabled this technique to become automated. This technique is now widely used on DNA extracted

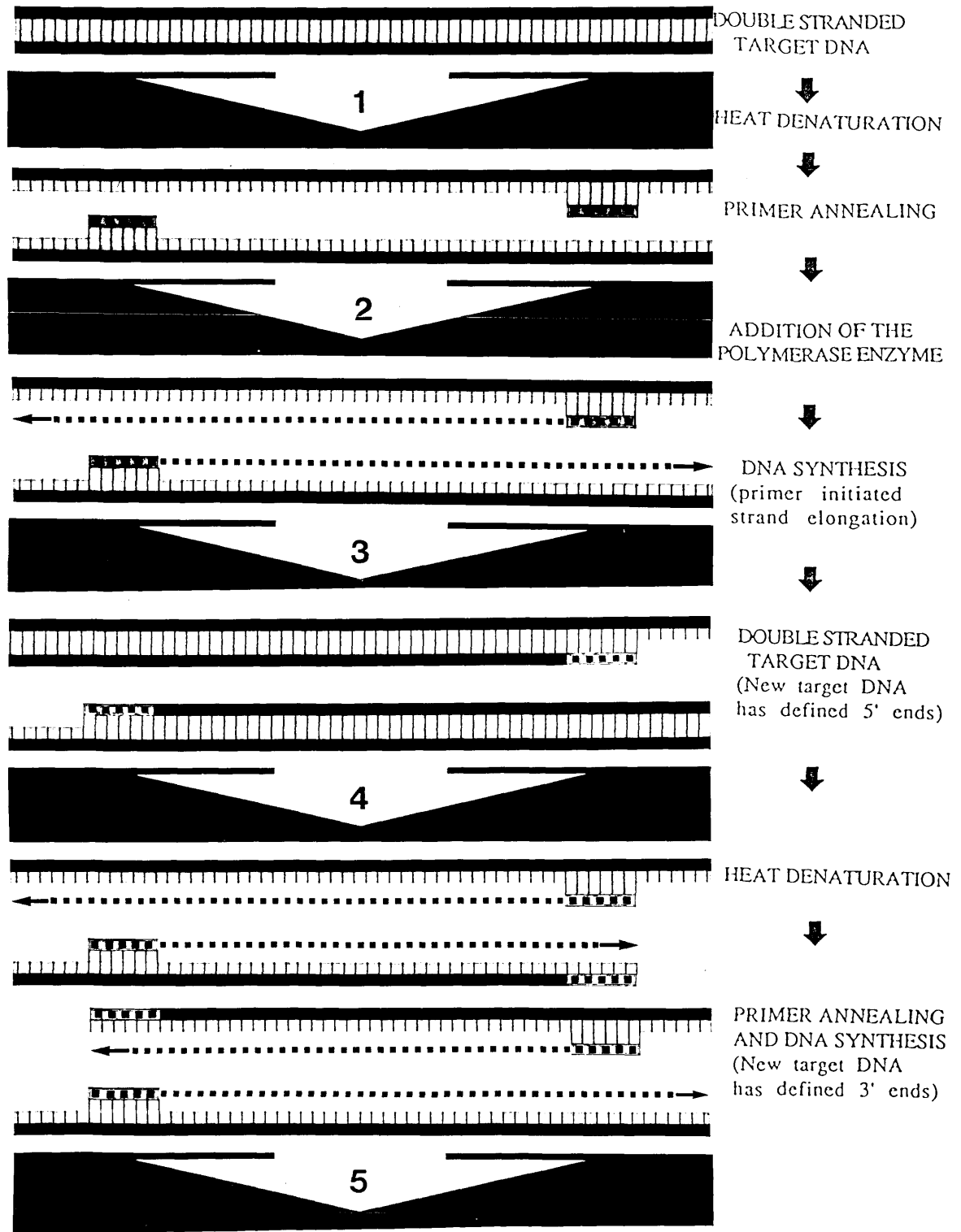


FIGURE 12 THE POLYMERASE CHAIN REACTION
(Saiki et al.,1985)

Each cycle of denaturation,annealing and synthesis generates new target DNA of a length defined by the distance between the two primers.The reaction uses either the large klenow fragment of DNA polymerase I or *Taq* polymerase for DNA synthesis,which occurs exponentially.

from blood (Kogan et al.,1987;Saiki et al.,1986;Petersen et al.,1988; Sheldon et al.,1986) and tissue samples (tumor,Vries et al.,1986; buccal epithelial cells,Lench et al.,1988; the root region of hair,Higuchi et al.,1988) bacteriophage libraries (Friedman et al.,1988) and has even been applied to DNA extracted from formaline-fixed (Impraim et al.,1987),paraffin embedded tissues (Shibata et al.,1988; Lai-Goldman et al.,1988).It has also been used for RNA amplification (Sarkar and Sommer,1988).

Selective enrichment of specific DNA,cDNA and RNA sequences using biotinylated probes,avidin and copper-chelate agarose has been described as an alternative procedure for concentrating target DNAs (Welcher et al.,1986).Biotinylated DNA or RNA is used as a hybridisation probe,complementary to the target DNA,in solution. Avidin is then added to label the hybrid molecules,which are then selectively bound to a column of cupric-iminoacetic acid/agarose beads.The sequences retained on the column are recovered in high yield by the addition of ethylenediamine tetracetic acid in the elution buffer.This method can be used in both subtractive enrichment, to isolate unusually expressed mRNA sequences and positive selection protocols,used to isolate specific sequences from gene libraries.

Other physical methods of sequence enrichment have been used,for example size fractionation.These procedures are however usually time consuming and less suited for the processing of large and numerous samples.

Within the last few years considerable attention has been given to indirect methods of covalent,non-radioisotopic labelling,in order to circumvent the problems inherent to radioactivity.Oligonucleotides were found to be ideally suited for this type of labelling strategy. Not only was complete separation of the modified oligonucleotide probes from all other reaction components easier than for large

polynucleotides (over 100 bases), it was also possible to control the number and position of labelling moieties. Label density could thus be maximised (Urdea et al., 1988). It was also thought that indirect non-radioactive labelling systems might be another way of significantly improving the detection limits of labelled oligonucleotides.

Most non-radioactive labels are difficult to incorporate directly during chemical synthesis of oligonucleotides since they are not stable in the reagents employed for synthesis and /or deprotection. As a result appropriately blocked nucleophilic "linkers" such as alkyl-sulphydryls or amines (see figure 13.) have been incorporated during the solid support synthesis of the DNA. More unusual linkage groups have been thiols, (Connolly and Rider, 1985) dialdehyde derivatives and uridyl residues (Agrewal et al., 1986). After deprotection and purification, these sites have been used to introduce a whole range of nucleophile specific labeling reagents, some of which are listed in table 5.

The detection limits of many labeling systems were estimated through simple sandwich hybridisation assays, using complementary probe and target oligonucleotides, by Urdea et al. (1988). The results demonstrated that enzymes such as horseradish peroxidase and alkaline phosphatase were the superior reporter groups, providing sensitivities nearly equivalent to single ^{32}P moieties.

Generally one label is tagged onto each oligonucleotide probe for detection. Recently however a method was developed for attaching several reporter groups at the 3' end of oligonucleotide probes using terminal deoxynucleotidyl transferase (Kumar et al., 1988). Oligonucleotides were tailed using commercially available nucleotide triphosphate analogues with a free primary amine. After tailing under optimal conditions, they were labelled with either biotin or

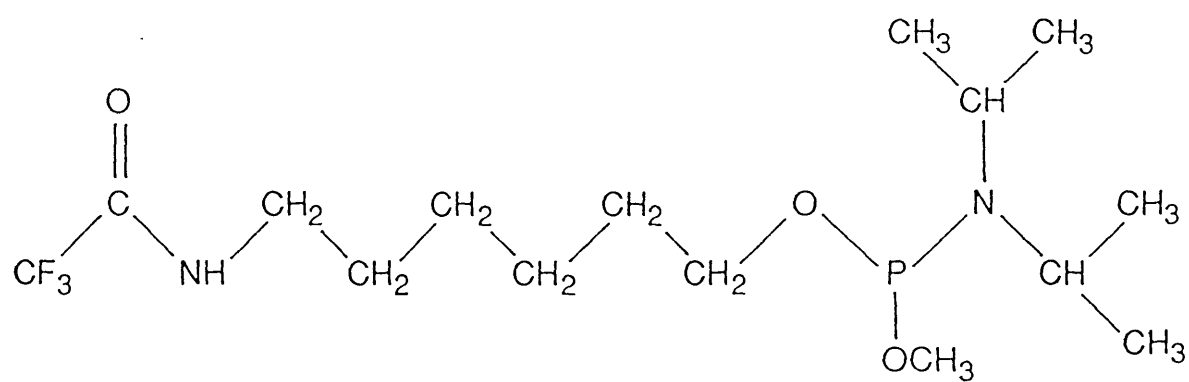


FIGURE 13 CHEMICAL STRUCTURE OF AMINOLINK II
(Applied Biosystems Ltd.,reproduced with permission)

fluorescein. In each case detection limits 2.5 times more sensitive than the limits found in previous reports for ^{32}P could be obtained.

1.8

Summary and aims

Gene cloning can probably be stated as being the key to the development of modern molecular biology and gene diagnosis. Together with the discovery of restriction enzymes and nucleic acid hybridisation techniques, cloning enabled the development of new methods of accurate DNA based diagnosis. These methods soon became established as useful alternatives to the more traditional methods, based on clinical symptoms and the detection of abnormal or foreign proteins.

Not only is DNA based diagnosis more accurate in defining inherited genetic error, in that it can detect mutational changes even in the absence of expressed protein product, it can also reveal the presence of infectious pathogens, even when these are masked by products of the human immune response. At present numerous diseases can be diagnosed using this approach.

Over the past years attempts have been made to improve the filter based methods of gene diagnosis, the prime efforts having been focused on reducing the time, effort and cost of the reactions as well as the development of non-isotopic labelling techniques. One of the techniques which evolved was the sandwich hybridisation technique using DNA probes covalently bound to microgranular support systems rather than to nitrocellulose or other filters. By immobilising recombinant DNA fragments to the support, specific target DNAs could be isolated from the genomic pool of nucleic acid sequences. These could be detected without the need for autoradiography. The sandwich assay has since enabled the detection of numerous infectious disorders.

With the increasing availability of new instrumentation, oligonucleotides soon became an important tool for molecular biology and human diagnostics. Synthetic oligonucleotides have made it possible to create purely man-made genes, to isolate genes, to create site-specific mutations in nucleic acids and to detect single base mutations responsible for human genetic diseases. Many recombinant DNA probes were soon replaced by oligonucleotides as these could be synthesised quickly and provided increased sequence specificity upon hybridisation.

It soon became clear that automation of DNA based diagnosis was required for it to gain greater acceptance in the medical world. The sandwich assay lends itself to automation, but has so far not been adapted for the qualitative analysis of single base mutations in human genomic DNA using oligonucleotides.

The aim of this work has been to develop a sandwich assay using oligonucleotides, which could be used to detect single base changes in genomic DNA. Using oligonucleotides covalently bound to resins, an efficient and stable immobilisation strategy had to be established which would not interfere with hybridisation to target DNA. The sensitivity of the assay had to be sufficient to detect single copy genes using labeled oligonucleotide probes. Furthermore the original assay procedure described by Dunn and Hassel (1977) had to be adapted to accommodate the possibility of differential hybridisation with pairs of oligonucleotide probes complementary to the normal and mutated gene sequence.

No reported attempt has previously been made to immobilise oligonucleotides in this way for the purpose of specific hybridisation to single copy genes. It was therefore necessary to find a support system which had a low affinity for non-specific adsorption of

oligonucleotides and one which suited the optimum immobilisation strategy for oligonucleotides in this assay. Different resins and coupling procedures were tested. It was also important to establish the effect oligonucleotide probe length had on the mentioned parameters as well as on hybridisation to the target DNA.

Two inherited gene disorders were chosen as a working model for the development of this assay, the sickle mutation of the beta-globin gene and the alpha-1-antitrypsin gene mutations found in association with the Z and S Pi type.

The sickle mutation is characterised by a single base change in the sixth codon of the beta-globin gene resulting in the loss of a Dde I and Mst II restriction site. It is also characterised by the substitution of a glutamic acid for a valine, leading to the production of HbS found in sickle cell disease. The mutation has been used as a model system for previous sandwich hybridisation work and was chosen primarily for this reason.

The two base changes of the alpha-1-antitrypsin gene also lead to aminoacid substitutions, however there are no known changes in restriction recognition sites. Both mutations have been associated with deficiencies of the alpha-1-antitrypsin protein, which often leads to severe clinical symptoms. Diagnostic procedures for the recognition of these mutations, using DNA probes have been labour intensive and cumbersome.

Oligonucleotides, to be used as labelled detector probes, had to be designed for both the normal and mutant genotypes at the site of mutation for the two genes. As part of the aim of this work, the possibility of converting the labeling strategy of these detector probes from a radioactive to a non-radioactive one, was to be approached. This would not only render the probes more stable but would also provide a safer method of gene detection.

The sandwich hybridisation assay proposed is based on the use of two non-overlapping and non-complementary oligonucleotide probes. One of the probes is immobilised to the solid support and serves to capture the target DNA (or the clinical sample). The other probe is labeled and used to identify the presence or absence of the target DNA through a hybridisation reaction. By running two hybridisation experiments in parallel, one with an oligonucleotide detector probe coding for the normal, and one for the mutated gene sequence, differential hybridisation to the same target DNA should be possible. The differential melting properties of the detector probes should thus indicate target genotype.

It was hoped that the development of this assay would not only assist in establishing a new, more efficient diagnostic procedure for the detection of single base changes, but might also serve as an improvement to the traditional sandwich assay, used for the quantitative analysis of infectious disorders. Such an assay system might also facilitate the development of new techniques for prenatal diagnosis, the analysis of tumors, and the diagnosis of acquired disorders.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

All chemicals with the following exceptions were Analar grade supplied by British Drug Houses (BDH), Poole, England. Sodium chloride, Trizma base, Trizma hydrochloride, 2(N-morpholino)ethane sulfonic acid (MES), urea, polyvinylpyrrolidone, dithiothreitol (DTT) Sigma, phenol and spermidine were supplied by the Sigma Chemical Co. St. Louis, Missouri, U.S.A. Ficoll and dextran sulphate were from Pharmacia, Uppsala, Sweden. Oligonucleotide synthesis chemicals were obtained from Rathburn Chemicals Ltd., Peebleshire, Scotland.

2.1.2 Enzymes

The enzymes were predominantly supplied by GIBCO Bethesda Research Laboratories (BRL), Paisley, Scotland: Bam HI, Hae III, Dde I, T4 Polynucleotide Kinase and T4 DNA ligase (isolated from T4 am N82-infected E. coli cells), .Terminal Deoxynucleotidyl Transferase (isolated from calf thymus), S1 Nuclease (isolated from Aspergillus oryzae). Restriction buffers were included with every shipment of enzyme. These were core buffers of high medium and low salt concentrations optimised for enzyme. Hind III and DNA polymerase I (the large Klenow fragment from DNA polymerase isolated from E. coli) were obtained from Pharmacia, Uppsala, Sweden. Horse radish peroxidase and Pst I were purchased from Boehringer Corp., Mannheim, West Germany. RNase A, lysozyme and calf intestinal alkaline phosphatase (CAP) were obtained from Sigma. DNA polymerase from Thermus aquaticus (Taq polymerase) was bought from Perkin Elmer Cetus Corporation,

Norwalk, Connecticut, U.S.A. and from New England Biolabs, Beverly, Massachusetts, U.S.A.

2.1.3 Isotopes

The isotopes (gamma-32 P dATP, 3000 Ci/mmol; alpha-32 P dCTP, 3000 Ci/mmol) were supplied in aqueous solution by New England Nuclear (NEN), Boston, Massachusetts, U.S.A.. Adenosine 5'-triphosphate of high specific activity (7000 Ci/mmol) was obtained from ICN Radiochemicals, Irvine, California, U.S.A.

2.1.4 Nucleic Acids

Salmon sperm DNA was from Sigma. Calf thymus DNA was from Koch Light Laboratories Ltd., Slough, England. Deoxyribonucleotides, PUC 8, lyophilised PUC 13 Bam HI, ϕ X174 RF DNA and bacteriophage lambda DNA were bought from GIBCO BRL, Rockville, Maryland, U.S.A.. Hexadeoxyribonucleotide primer [p(dN)6] was obtained from Amersham international, Amersham, England. Transfer ribonucleic acid (tRNA) from bakers yeast was supplied by Boehringer. Phosphoramidites and tetrazole were obtained from Cruachem, Glasgow Scotland. Controlled pore glass (CPG) supports for oligonucleotide synthesis were bought from Pharmacia. Aminolink II was supplied by Applied Biosystems Ltd., Foster City, C.A., U.S.A.

2.1.5 Non-radioactive Detection of Nucleic Acids

"Long chain" N-hydroxysuccinimidyl biotin was provided by Pierce Chemicals, Oud-Beijerland, Netherlands. Streptavidine-Horseradish peroxidase conjugate (SA-HRP) was obtained from GIBCO-BRL.

O-phenylenediamine dihydrochloride, 3,3',dianisidine, 3,3',5,5'tetramethyl benzidine dihydrochloride dihydrate (TMB) and p-phenylene diisothiocyanate were from Aldrich Chemical Co.Ltd., Gillingham,Dorset,England.Hydrogen peroxide (supplied as a 30% v/v solution) and imidazole were from Sigma.

2.1.6 Bacteriological Reagents

Agar, yeast extract and tryptone were supplied by Difco Laboratories, Detroit, Michigan. 5-Bromo-4-chloro-3-indolyl beta-D-galactoside (X-gal) and isopropylthio beta-D-galactoside (IPTG) were bought from GIBCO-BRL. Ampicillin and Chloramphenicol were obtained from Sigma. Sterile 90 mm Petri dishes were from Sterilin Ltd., Teddington, England.

2.1.7 Nucleic Acid Purification and Chromatography

Sephadex and NAP-10 columns were from Pharmacia. The Mono Q (ion exchange) HR 10/10 column for FPLC purification was also from Pharmacia. The ion exchange HPLC column was from Whatman Ltd. Maidstone, Kent, England. "Nensorb-20" purification cartridges and "Nensorb Prep." columns (NLP-028 Nensorb Prep cartridges) were bought from Du Pont, Boston, Massachusetts, U.S.A.. Centricon -10 microconcentrators were from Amicon Corporation, Danvers, Massachusetts, U.S.A..

2.1.8 Electrophoresis, Transfer and Hybridisation

Ultrapure agarose and low melting point agarose (Seaplaque) were supplied by BRL. Nu-sieve agarose was from ICN Biomedicals Ltd. and

manufactured by FMC Bioproducts, Rockland, ME., U.S.A. Nitrocellulose filters were from Schleicher and Schull, Dassel, West Germany. Zeta probe membranes (cationized nylon membrane) were bought from Bio-Rad Laboratories. Whatman chromatography 3mm paper and DE-81 filter discs were from Whatman. Nuclease-free bovine serum albumin (BSA) was purchased from Pharmacia.

2.1.9 Photography and Autoradiography

The U.V. light source was a short wave transilluminator from Fotodyne, New Berlin, Wisconsin, U.S.A. Phenol developer and Hypam fixer were from Ilford, Basildon, England. RX medical X-ray film was from Fuji Photo Co. Ltd., Tokyo, Japan. X-Omat XAR -5 X-ray film was from Eastman Kodak, Rochester, New York, U.S.A. Okamoto film cassettes and screens were from X-Ograph Ltd., Malmesbury, Wiltshire, England. FP-4 film was obtained from Ilford, London

2.1.10 Resins and Crosslinking Reagents

Glass microfibre filters (GF/C) were purchased from Whatman. 1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate, 95%, 1,4-butanediol diglycidyl ether, 95% and 2-aminothiophenol were purchased from Aldrich. Sephadexes, Sephacryl S-1000, Oligo (dT) celluloses and oligo (dU) cellulose, Epoxy activated Sepharose 6B and AH Sepharose 4B were bought from Pharmacia (registered trademarks of Pharmacia).

2.2 Bacterial Strains and Nucleic Acids

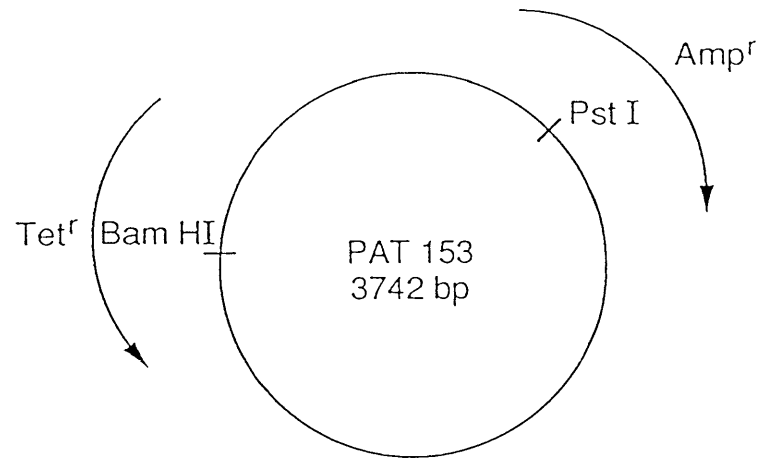
2.2.1 Bacterial Phage Strains

The globin plasmid recombinants were propagated in E. coli strains HB101 (Boyer and Roulland-Dussoix, 1969). The genotype of HB101 was: F⁻, hsdS20, rB⁻, rec A⁻, supE44, ara-14, gal K-2, lac Y1, proA2, rps L20, str r, xyl-5, mtl-1.

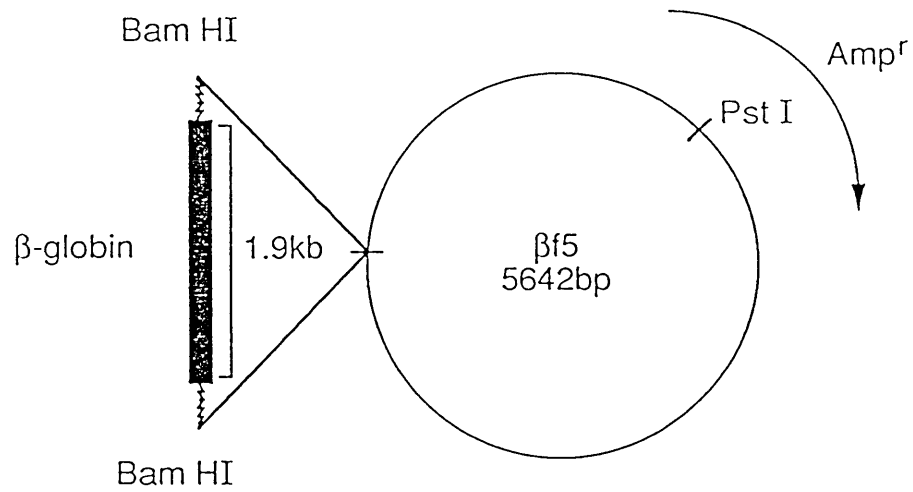
The plasmid Hbf 5 was a pAT153 (Twigg and Sherratt, 1980) derived plasmid that contained a 1.9 kbp restriction fragment of the 5' end of the human beta-globin gene (Maingon, 1982). A simplified restriction map of this plasmid is shown in figure 14. The sickle mutated, 1.9 kbp beta-globin gene was derived from a pBR322 recombinant plasmid, Hbl-S (Woodhead 1986). Hbl-S was a kind gift from L. Woodhead in this laboratory. A simplified restriction map is shown in figure 15. Both gene inserts, containing either the normal or the sickle mutated beta-globin gene, can be excised from the vector DNA through Bam HI restriction digestion.

Plasmids pTG 603 (M-sequence) and pTG955 (S-sequence) were pBR322 derivatives containing the human alpha-1-antitrypsin cDNA sequence. The gene insert of pTG603 (contained within two Pst I restriction sites) codes for the entire cDNA sequence minus 12 nucleotides from the 5' end, whereas the insert of pTG955 (bound by Nde I and Pst I restriction sites) contains an incomplete cDNA sequence of the alpha-1-antitrypsin gene. The plasmid pTG955 was created by site directed mutagenesis of pTG603 at the S mutation site. These plasmids were a kind gift from M. Courtney, Transgene SA, Strasbourg.

All bacterial stocks were stored as exponential cultures either in 50% v/v glycerol at -20°C or in 20% v/v glycerol at -70°C.

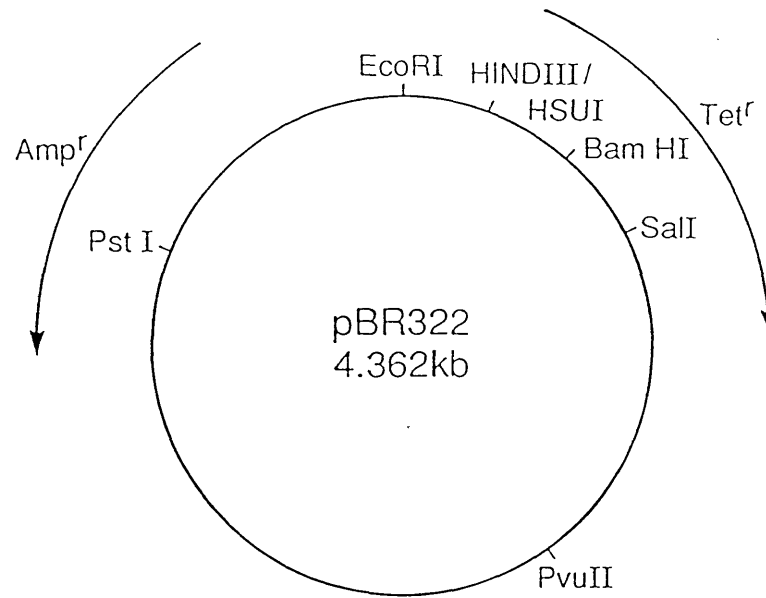


A. Plasmid PAT 153 (-PBR322 derivative)

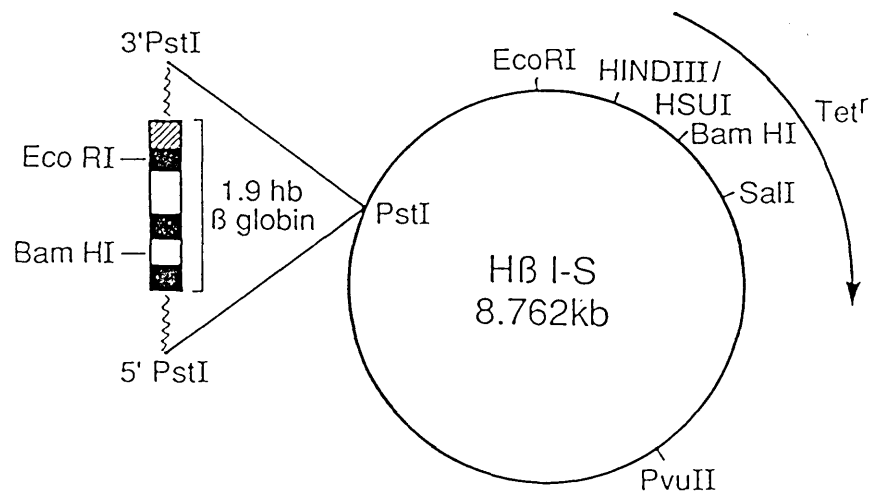


B. Recombinant Plasmid Hβ-f5

FIGURE 14 Restriction Enzyme Maps



A Plasmid PBR322



B Recombinant plasmid HB I-S

FIGURE 15 Restriction Enzyme Maps

2.2.2 Oligonucleotides and Patient Blood Samples

With the exception of the oligonucleotide containing 2-aminoadenine all other oligonucleotide sequences were synthesised in the laboratory on a Gene Assembler (Pharmacia), or using the Manual Assembler (Cruachem). The 20mer beta-globin oligonucleotide containing one 2-aminoadenine moiety was synthesised by A. Chollet, at the Glaxo Institute for Molecular Biology S.A., Switzerland.

Patient blood samples AA (globin normal), AS (heterozygote for the sickle cell mutation site) and SS (homozygote sickle) for the beta-globin gene were a kind gift from the Haematology department, Charing Cross Hospital, London. The blood sample containing a normal genotype for the alpha-1-antitrypsin gene was obtained from a member of staff from Charing Cross Hospital, London.

2.3 METHODS

2.3.1 Preparation of DNA

2.3.1.1 Preparation of Human DNA

DNA was extracted from human peripheral blood cells based on a method originally devised by Kunkel et al. (1977). Fresh blood was collected in heparinised tubes or in vials containing EDTA, so that the final concentration was 5mM. Heparin and EDTA acted as anticlotting agents and made further storage of the blood at -20°C or -70°C possible.

To 10 ml volumes of blood maintained at approximately 4°C , 90 ml of the following lysis buffer was added: 0.32 M sucrose, 10 mM Tris-HCl

(pH. 7.5), 5 mM magnesium chloride, 1% v/v Triton X-100. The sample was gently mixed and then pelleted by centrifugation in a Sorval GSA (HB-4) rotor (pre-cooled) at around 4°C for 10 min. at 10,000 rpm (16,322 x g av). Using a plastic sterile pipette the pellet was resuspended in 4.5 ml. of 0.075 M NaCl, 0.024 M EDTA (pH. 8.0). SDS and proteinase K were then added to a final concentration of 0.5% (w/v) and 200 g/ml respectively. The Proteinase K digestion was incubated either at 37°C overnight or at 55°C for 2 hours. The samples were subsequently extracted three times with phenol/chloroform and then precipitated with ethanol. The precipitates were dissolved in TE overnight at 4°C. DNA concentrations were estimated by O.D. 260 nm measurements using a Pye-Unicam SP-8 400 UV spectrophotometer.

2.3.1.2 Preparation of Plasmid DNA

Plasmid DNA was prepared according to a method described by Ish-Horowitz and Burke (1981). E. coli containing pBR322 or pAT153 derived plasmids were grown in L-broth (5g/litre yeast extract, 10g/litre tryptone, 5g/litre NaCl, pH. 7.2), whereas those containing pUC8 derived plasmids were grown in 2 x YT broth (10g/litre yeast extract, 16g/litre tryptone, 10g/litre NaCl). One colony or 10 ul of a suitable glycerol stock was used to inoculate 10 ml broth containing an appropriate antibiotic (50 ug/ml ampicillin or tetracycline). The culture was incubated overnight on a rotary incubator at 37°C. Using 10 ml of this starter culture, 1 litre fresh medium was inoculated and incubated until the OD 600 reading reached 0.4, when 170 ug/ml chloramphenicol was added. Reincubation occurred at 37°C overnight with shaking. The culture was then pelleted by centrifugation at 5000 rpm (2560 g av) in a GSA rotor for 5 min at 4°C.

The pellet was drained and resuspended in 20 ml of the

following lysis buffer: 50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 5 mg/ml lysozyme. After a 10 min incubation at room temperature 40 ml 0.2 M NaOH, 1% w/v SDS was added, a further incubation occurring on ice for 10 min with gentle shaking. The cell debris and high molecular weight DNA were precipitated upon the addition of 20 ml 3 M potassium acetate, pH 4.8, which was mixed vigorously into the suspension. A 30 min incubation on ice was followed by pelleting by centrifugation at 7000 rpm (5019 g av) in the GSA rotor at 4°C for 10 min. To the supernatant an equal volume of isopropanol was added and left for 10 min at room temperature. The DNA was pelleted out by centrifugation at 10 000 rpm (10240 g av) and 4°C for 10 min. The supernatant was discarded and 30 ml TE added to dissolve the pellet through gentle aspiration using a sterile pipette. To the homogenate 0.5 mg/ml ethidium bromide and 1 g/ml caesium chloride was added. Any remaining debris was cleared with a centrifugation at 12 000 rpm (14,740 x g av) at room temperature for 30 min.

The gravity of the solution was adjusted to 1.56 prior to transfer into 36 ml cellulose nitrate centrifuge tubes. The tubes were spun at 50 000 rpm (226,200 x g av) in a Sorval SA 600 rotor for 16 hours at 18°C.

Under long wave U.V. illumination the lower DNA band was extracted using a sterile syringe. The ethidium bromide was removed with an equal volume of isopropanol and TE saturated with caesium chloride. The plasmid DNA was dialysed overnight at 4°C against TE buffer and extracted 3 times with phenol/chloroform, ethanol precipitated and resuspended in TE.

DNA concentrations were determined by O.D. 260 nm measurements using a spectrophotometer.

2.3.1.3 Oligonucleotide Synthesis

Oligonucleotides synthesised semi-manually were assembled by the phosphotriester method described by Sproat and Gait (1984). The chemical basis of this reaction is illustrated in figure 16. The apparatus for synthesis was a one column manual model oligo-assembler (Cruachem). The system relied on Argon pressure (4 p.s.i.) to maintain a constant flow rate through the column. The support resin used was 50 mg of the appropriate functionalised long chain alkylamine controlled pore glass (CPG) support (0.2 μmol of bound 3'-terminal nucleoside). For each cycle of nucleotide addition 26.6 μmol of the appropriate nucleotide monomer was required. The extent of coupling could be monitored at each cycle after the acid deprotection step by collecting the liberated dimethoxytrityl cation and measuring its O.D. 495 nm in the presence of acid (60 % tricarboxylic acid or 60% aqueous perchloric acid /ethanol 3:2 v/v). The reading was multiplied by 25 to give total absorbance units.

Oligonucleotides synthesised automatically were assembled by the phosphite-triester method developed by Matteucci and Caruthers (1981). The overall operation of this method is very similar to the phosphotriester method except for the addition of the oxidation step to convert the phosphites to the phosphotriesters. The phosphite-triester chain assembly scheme is illustrated in figure 16. Synthesis was carried out on a Pharmacia Gene-Assembler, which relies on a peristaltic pump and a chemically inert pump hose to control the flow rate. Phosphoramidites, tetrazole and acetonitrile were kept under an argon atmosphere to protect them against water moisture. The columns applied were of controlled pore glass containing 1.3 μmol of the starting nucleoside. For each cycle 30 μmol of the appropriate nucleotide monomer was required. Coupling efficiency was monitored and

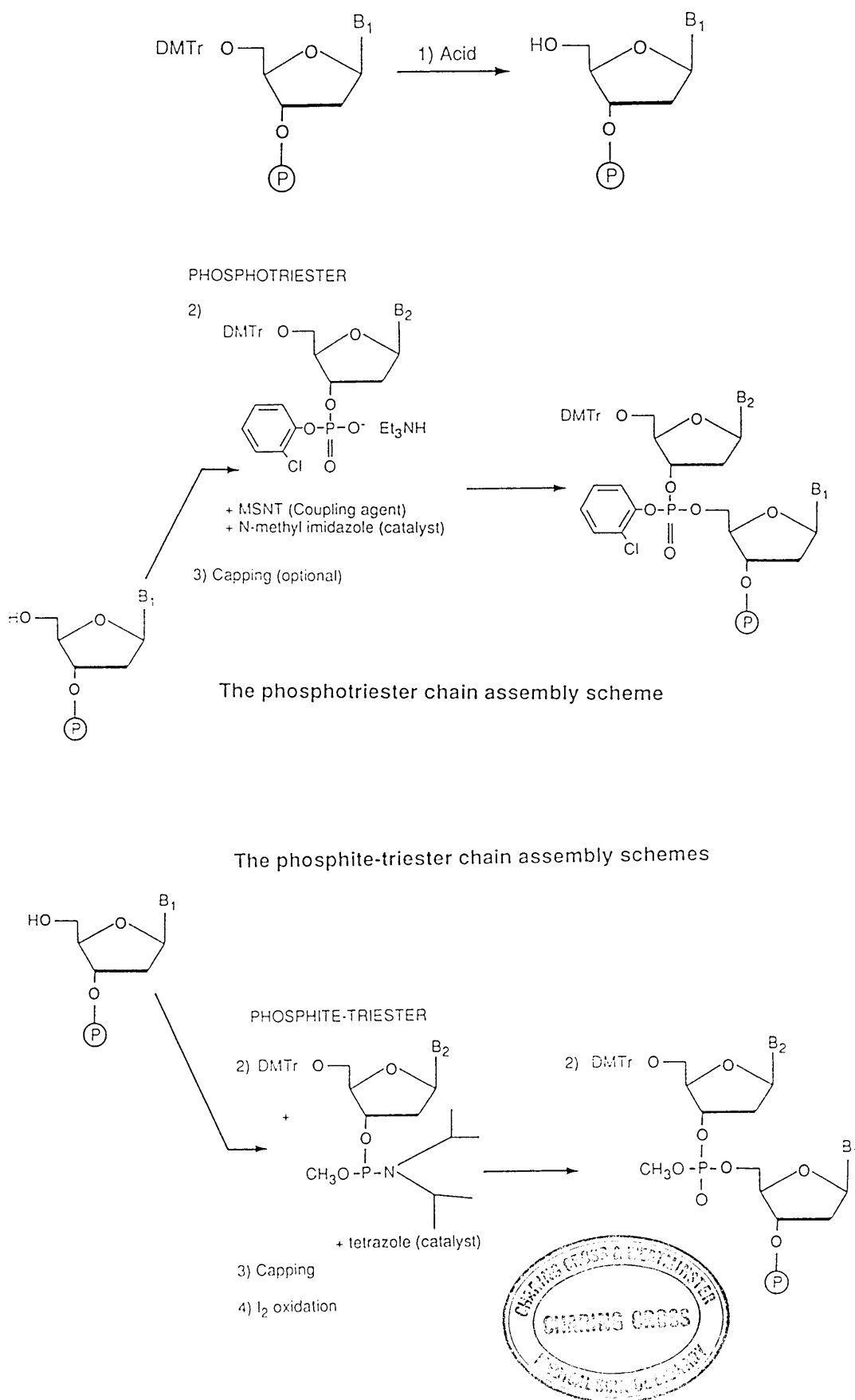


FIGURE 16 The phosphotriester and phosphite-triester chain assembly schemes.

recorded automatically by a flow through photometer operating at O.D. 436 nm.

Cleavage and deprotection of the assembled product from the solid support for both methods of synthesis occurred through a 16 hour incubation in concentrated ammonia at 50°C. If further purification of the oligonucleotide did not rely on the presence of the dimethoxytrityle cation protecting the 5'-hydroxyl group, then the removal of this group was achieved by treatment in acetic acid / water (8:2 v/v) at room temperature for 15 min. Rapid removal of the acetic acid and contaminating groups was attained by gel filtration through a "Nap-10" column. An alternative method involved exposing the immobilised oligonucleotide prior to cleavage and deprotection, to a short treatment of DCA in DCE until all the cation was released.

2.3.2 Sub-cloning of Plasmid DNA

Fragments purified for sub-cloning were size fractionated by agarose gel electrophoresis and were extracted using a "Biotrap extractor" (electrophoretic extraction). Final purification through a "Nensorb-20" column ensured removal of contaminants (agarose derived sulphydril groups).

Beta-globin gene insert (coding for the sickle cell disease mutation), with Bam HI staggered ends was ligated into linearised PUC 13. This vector contained Bam HI ends, which had been phosphatased to reduce self ligation. The ligation was set up at a molar ratio of 3:1, insert:vector. The vector and insert DNA were incubated for at least 4 hours in a 20 ul reaction volume. Contained in the reaction buffer were: 10 units of ligase and 12mM magnesium chloride, 5mM DTT, 1 mM ATP, 70 mM Tris-HCl pH. 7.6 at 15°C. Control ligations with phosphatased vector alone were also prepared.

The ligation mixes were transformed into HB101 by using 100 μ l of a HB101 starter culture (bacterial glycerol stock) to inoculate 10 ml of L broth. The cells were grown with vigorous shaking at 37°C until they reached an O.D. 550 nm of 0.5. The culture was chilled on ice for 10 min and then centrifuged at 4°C for 5 min. The supernatant was discarded and the pellet resuspended in 5 ml ice-cold, sterile solution of 50 mM calcium chloride and 10 mM Tris-HCl (pH 8.0). The cells were then incubated on ice for 15 minutes and recentrifuged as above. The supernatant was again discarded and the cells were resuspended in 1 ml of 50 mM calcium chloride, Tris-HCl (pH 8.0). Aliquots of 200 μ l were mixed with 20-100 ng of the transforming DNA and stored over night at 4°C. (12-24 hours).

The following day the cells were exposed to heat shock treatment performed at 42°C for two min. The transformed bacteria were left to recover and express antibiotic resistance during a 30 min incubation (without shaking), at 37°C with an added 1 ml of L-broth. Each tube was subsequently plated out onto L-broth media containing 1.5 % agar, 50 g/ml ampicillin, 0.004 % w/v X-gal, 0.0008 % w/v IPTG and incubated at 37°C overnight.

The transformation control of supercoiled PUC 13 was plated out in the same fashion. The expected white recombinant colonies were used to inoculate mini-cultures, 5 ml of L-Broth containing 50 g/ml ampicillin, at 37°C overnight with shaking. Using 1 ml of a culture pelleted in a microfuge (MSE microcentaur), the bacteria were resuspended in 100 μ l of 50 mM glucose, 25 mM Tris-HCl, pH.8.0, 10 mM EDTA, 5 g/ml lysozyme. The suspension was left for 5 min at room temperature. To this 200 μ l 0.2 M NaOH and 1% SDS were added. The suspension was gently shaken and incubated on ice for 10 min. Finally 150 μ l 3 M potassium acetate pH.4.8 was added, the sample was vortexed and left on ice for a further 10 min. The cell debris was pelleted by

centrifugation in the microfuge and the supernatant containing the DNA phenol/chloroform extracted followed by ethanol precipitation. The resuspension of the pellet was in TE buffer.

The ligation products were analysed for the presence of the correct insert (1.9kb fragment), by restriction digestion, using the restriction enzyme Bam HI and agarose gel electrophoresis. Recombinants were stored as exponential cultures in 50 % v/v glycerol at -20°C.

2.3.3 General methods

2.3.3.1 Extraction and Purification of Nucleic Acids

DNA samples were purified from contaminating proteins either by phenol/chloroform extraction or by running them through a "Nensorb-20" purification cartridge.

Phenol/chloroform extraction was ideally suited for large DNA fragments (whole blood DNA, intact plasmid DNA and fragments larger than 500bp in length). An equal volume of phenol saturated with 10mM Tris(hydroxymethyl)amino methane (Tris-HCl), 1 mM ethylene diamine tetra-acetic acid (EDTA), pH 8.0 (TE buffer) was added to the DNA and mixed thoroughly. The aqueous layer was separated out by centrifugation for 10 min either at 11,000 x g av in an eppendorf microfuge (MSE microcentaur) or at 14,481 x g av (10,000 rpm) in a Sorval SA 600 rotor. Traces of remaining phenol were extracted by repeating the procedure with chloroform:isoamylalcohol.

"Nensorb-20" purification cartridges were used primarily for the purification of small DNA fragments (>500 bp in length). They were used as specified by the manufacturers (Du Pont), however final extraction of the bound DNA from the column was in 20% ethanol and in a volume of less than 500 ul.

Ethidium bromide was removed from DNA samples by extracting three times with an equal volume of water saturated butan-1-ol.

2.3.3.2 Concentration of Nucleic Acids

The concentration, desalting and exchanging of buffer solutions from DNA samples was fragment length dependent. Large DNA fragments (greater than 500kbp and less than 10,000kbp) were precipitated by the addition of 0.1 volumes 3M sodium acetate pH.4.5 and 2.5 volumes ethanol. The samples were either chilled over night at -20°C or for 1 hour in a dry CO_2 /ethanol bath (-70°C). The DNA was pelleted by centrifugation for 10 min in a microfuge or at 10,000 rpm in a SA 600 rotor. The pellet was rewashed in 70% ethanol and repelleted. The final pellet was dried under vacuum and redissolved in a suitable buffer. The high molecular weight DNA, such as human genomic DNA was extracted directly with a closed pasteur pipette and rinsed in 70 % ethanol. The precipitate was allowed to dry in air before dissolving in a buffer.

Short DNA fragments (less than 500kbp) and oligonucleotides were concentrated by exchanging the buffer solution to 20% ethanol, which then could be removed by evaporation under vacuum. Exchanging the buffer solution was achieved either by gel filtration, using Sephadex G25 columns or Nap 10 columns, by using "Nensorb 20" purification cartridges, or by using microconcentrators, Centricon-10 (Amicon). Table 6. in section 2.3.8.1, compares the efficiency of gel-filtration and affinity column purification strategies, for short DNA fragment lengths.

<u>Properties</u>	<u>Gel filtration</u>		<u>Spun Columns</u>	<u>"Nensorb" affinity chromatography</u>
	<u>Sephadex G-25</u>	<u>G-50</u>		
optimum size	>100mer	<100mer	depends on the Sephadex used	<100mer
% 32 P contaminants	0-10	0-10	50-80	0-10
Elution volume	500-800ul	500-800ul	200-500ul	200-500ul
Probe loss	negligible	negligible	negligible	20-30% for DNA's >100mer

TABLE 6 PURIFICATION METHODS TESTED FOR THEIR EFFICIENCIES
IN REMOVING UNINCORPORATED 32 P FROM OLIGONUCLEOTIDE
LABELLING REACTIONS

2.3.3.3 Restriction Endonuclease Digestion.

Restriction digests were set up with suitable core buffers supplied by the manufacturer, 100 ug/ml nuclease free BSA, DNA (0.1-0.2 mg/ml) and the appropriate restriction enzyme. Plasmid DNAs (pTG603, pTG955, Hbf 5, Hbl-S and Hb2-S) were digested using 1 unit of restriction enzyme per ug of DNA, with an incubation of 1-2 hours. The restriction enzymes used for the reactions performed were Pst I, Bam HI, Hae III, Dde I and Nde I. Human genomic DNA samples were digested with 5 units of restriction enzyme (Pst I, Dde I) per ug DNA and an incubation of 4-16 hours. The temperatures used for incubation were generally around 37°C or as recommended by the manufacturers.

2.3.3.4 Random Shearing of High Molecular Weight DNA

Calf thymus DNA and salmon sperm DNA were sheared by sonication to an approximate sizes of 300bp and 500bp respectively. The DNA was dissolved in water at a concentration of 5 mg/ml. Sonication was carried out on ice for 10 x 30 seconds using an MSE soniprep 150. The ethidium stained agarose gel, in figure 17, demonstrates the size of the sonicated calf thymus DNA.

2.3.3.5 Gel Electrophoresis of Large DNA Fragments

Agarose gel electrophoresis was used for size fractionation and molecular weight analysis. It was required for Southern transfer and direct gel hybridisation procedures, also for extracting and purifying specific DNA fragments.

Agarose gels used to separate large DNA fragments (<1000 bp) were prepared as a 1% w/v suspensions dissolved either in E buffer

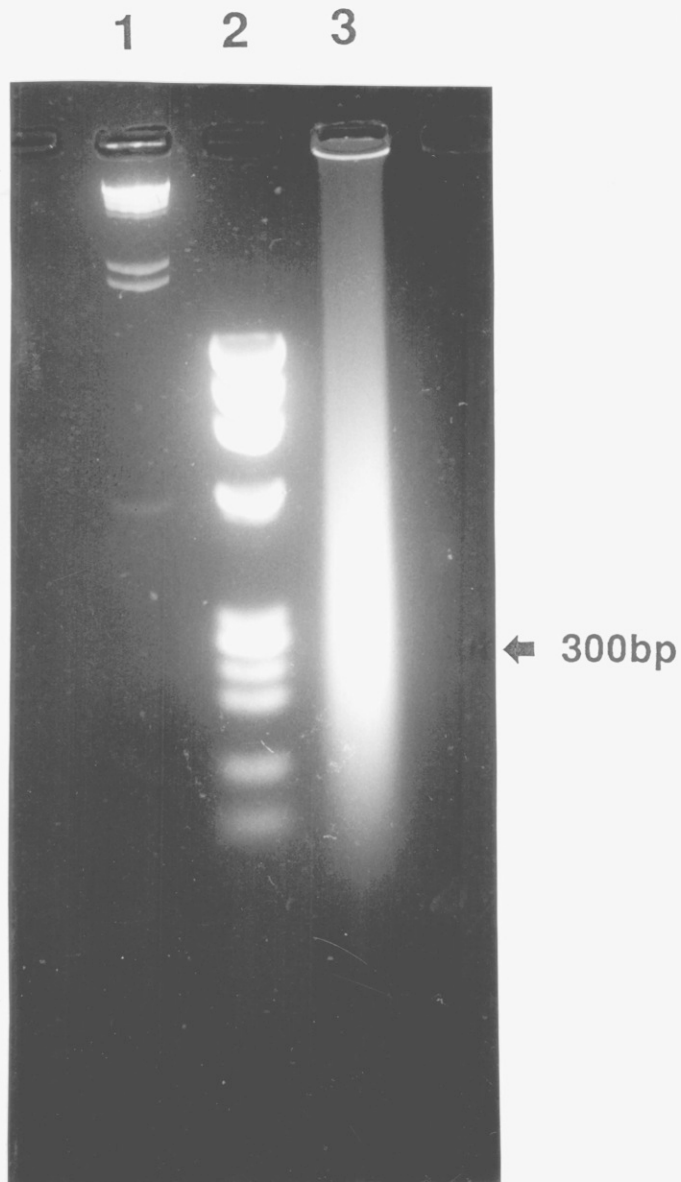


FIGURE 17 AN ETHIDIUM STAINED AGAROSE GEL DEMONSTRATING THE SIZE OF THE SONICATED CALF THYMUS DNA

Lanes 1 and 2 contain λ Hind III and ϕ X174 Hae III markers respectively. Lane 3 contains 1ug of the sonicated calf thymus DNA. The results show that the majority of the DNA fragments formed are between 200 bp and 500 bp long. The average size was taken to be approximately 300bp.

(40 mM Tris-HCl, 20 mM sodium acetate, 2 mM EDTA, pH 7.6) or TBE buffer (Tris base, Boric acid, EDTA). The agarose was dissolved through boiling for three minutes in a microwave oven. Electrophoresis was performed in the same buffer, however containing 1 g/ml ethidium bromide. Small DNA fragments (>1000 bp) were separated in agarose gels prepared with a higher percentage of agarose. In these gels the agarose used over and above the ordinary concentration was of high refractive index (Nu-sieve agarose, FMC). This permitted a greater resolution of the small DNA fragments.

Denaturing agarose gels containing 50 mM NaCl and 1 mM EDTA, and run in an alkaline electrophoresis buffer of 30mM NaOH and 1 mM EDTA were used to separate single stranded DNA fragments (<100bp) such as those generated by the polymerase chain reaction. The samples of DNA to be analysed were generally taken up in 10-20 ul alkaline loading buffer containing 50 mM NaOH, 1 mM EDTA, 2.5% Ficoll (type 400; Pharmacia), and 0.025% bromocresol green.

Low melting point agarose gels (1-2%), were used for the separation of DNA fragments to be extracted from the gel for further manipulations, such as cloning.

Samples for all of the mentioned gels were loaded in 0.5% w/v sodium dodecylsulphate (SDS), 0.05% w/v bromophenol blue (BPB), 5 mM EDTA and 10% w/v Ficoll. The gels were subjected to electrophoresis (50 V), for several hours or until the tracking dye front reached a suitable distance. The DNA fragments were stained with ethidium bromide and visualised by illumination with short wave UV light. Photographs of the gels were made using FP-4 film.

2.3.3.6 Gel Electrophoresis of Small DNA Fragments and Oligonucleotides

Acrylamide gels were used for the separation as well as the purification of small DNA fragments (>100bp) and oligonucleotides. Fragment size determined the concentration of acrylamide used. Gels were made from a 30% w/v acrylamide/0.8% w/v N,N'-methylene-bis-acrylamide stock solution which was diluted to an appropriate concentration with TBE buffer. Denaturing gels were prepared in a similar fashion, with the final solution however containing 7 M urea.

The gels were polymerized by the addition of 60mg ammonium persulphate and 35 ul N,N,N'-tetramethylethylenediamine (TEMED) per 100 ml gel solution. The gels were pre-run for 30 min in the presence of TBE buffer and the samples were run with the same buffer for 1-4 hours at voltages between 150 and 250. For non-denaturing gels the loading buffer used was the same as that used to load agarose gels. For denaturing gels the loading buffer contained 80% deionised formamide, 5 mM EDTA (pH.7.0), 0.02% w/v bromophenol blue and 0.05% w/v xylene cyanol.

2.3.4 The Polymerase Chain Reaction

The enzymic amplification of specific target sequences in DNA was carried out using the method described by Saiki (1988). This reaction was performed either with Thermus aquaticus (Taq polymerase), a heat-stable DNA polymerase (developed by Perkin Elmer-Cetus Corporation), or the large Klenow fragment of DNA polymerase I.

Amplifications using the Taq polymerase were performed with 0.20-0.5 ug of template DNA, 50 mM potassium chloride, 10 mM Tris-HCl

(pH.8.4), 2.5 mM magnesium chloride, 1 uM of each sequence specific primer, 200 uM each deoxyribonucleotide triphosphate (dATP, dCTP, dTTP, dGTP), 5 ug of gelatin, and two units of Tag polymerase in a final volume of 50 ul. To each sample 30 ul of mineral oil was added to prevent evaporation.

Amplifications using the large Klenow fragment of Escherichia coli DNA polymerase I were performed with a buffer containing: 10 mM Tris-HCL (pH.7.5), 50 mM NaCl, 10 mM magnesium chloride, 1.5 mM deoxyribonucleotide triphosphate ((dNTP) each of all four was used) and 0.5 units/ul Klenow enzyme. The DNA constituents used were the same as those mentioned previously for Tag polymerase.

For amplification of the beta-globin gene the primers used (PCO3 and PCO4) were 20 bases in length and separated from one another by 110 bases.

The manual procedure recommended for heat blocks or water baths required an initial template denaturation step of 10 min at 94°C. Afterwards, a typical cycle profile using Tag polymerase consisted of: 3 min annealing at 55°C, 4 min extension at 72°C and 2 min denaturation at 94°C. Automatic amplification, using the Hybaid intelligent heating block, was set to shorter cycle times after the initial denaturation step. The annealing and elongation steps were left for 30 sec and subsequent denaturation was only for 1 min. Manual amplification was usually carried out for 25-30 rounds, whereas automatic amplification was left to run for 50 rounds.

The cycle used for Klenow based PCR amplification involved heating for 5 min. at 95°C (to denature the genomic DNA), centrifugation in a microfuge to remove the condensation and an annealing step for 2 min at 37°C. At the end of this period the Klenow enzyme was added and incubation was allowed to proceed at 37°C for another 2 min. This cycle was repeated with subsequent denaturations performed for 2 instead of

5 minutes. Fresh Klenow enzyme was added in each cycle.

Small aliquots of the reaction products (5-10 ul) generated through PCR amplification were routinely separated on a 2-3% agarose gel (1% non-modified agarose gel, 3% Nu-sieve agarose) under electrophoresis. The extent of amplification could then be determined visually under UV. illumination by ethidium bromide staining the gel.

2.3.5 Extraction of Specific DNA sequences and Oligonucleotides

Specific DNA fragments (<100bp) were routinely extracted from restriction digests, size separated through electrophoresis on low melting point agarose (1-2%). After visualisation of the ethidium bromide stained bands under UV, the agarose gel containing the DNA of interest was cut out using a sharp scalpel blade. The gel slice was placed in a "Biotrap" electrophoresis tank and immersed in a 1 x TBE buffer. The gel slice was then subjected to an electric current of 100 V for 2-3 hours. The eluted DNA was purified from contaminating sulphhydryl groups using a "Nensorb-20" affinity column. An OD 260 nm reading of absorbance was then made using a spectrophotometer to establish final DNA fragment yields.

DNA restriction fragments and plasmid inserts could also be extracted through fast protein liquid chromatography (FPLC). The digest mixtures were applied to a Mono-Q column, the maximum loading capacity of which was 2 O.D. 254 nm. Using a salt gradient of between 0.8-0.85 M KCl in 25mM Tris-HCl, pH.8.3 most DNA restriction fragments could be isolated from other fragments in a separation time of less than 2 hours. After desalting the extracted DNA fraction (through gel-filtration), an OD 260 nm reading could be made, to establish final yields. This method was ideally suited for the extraction of DNA sequences from large DNA preparations and also small DNA fragments

(less than 1000kbp long).

Oligonucleotides were also extracted from crude mixtures using affinity chromatography or methods involving extraction from electrophoretically separated DNAs.

The products of oligonucleotide synthesis were extracted from shorter incomplete coupling sequences in one of four ways. Prior to deprotection of the 5' end with acetic acid the oligonucleotide mixture could be purified in affinity purification columns ("Nensorb Prep" columns, by Du Pont). Only the tritylated oligonucleotides were (all those involved in the last coupling step) bound to the affinity resin and could be eluted by treatment with methanol.

Alternatively the oligonucleotide could be purified using FPLC and a Mono-Q ion-exchange column. A salt gradient between 0.4 M NaCl and 0.8 M NaCl in 1mM NaOH pH.12 run over 1 hour routinely purified oligonucleotides between 15 and 100 residues in length.

HPLC could also be used for the purification of oligonucleotide sequences. The method applied to the purification of 20mers used a Whatman Partisil 10-Sax ion-exchange column and a 0.001 M to 0.3 M (phosphate buffer) salt gradient, run over 1 hour in the presence of 60% formamide.

Finally, crude mixtures of oligonucleotides could be separated from the end products by electrophoretic separation through 10-15% (depending on oligonucleotide length) denaturing polyacrylamide gels. Typically one fifth of the synthetic product was purified on a single gel. The DNA was visualised by a technique known as UV shadowing. The gel was removed from the glass electrophoresis plates and was wrapped in cling film. When placed on a fluorescent sheet (such as an indicator t.l.c. plate) and illuminated with UV light, the band of interest (the final synthetic product), was seen as the darkest band positioned at the top of the gel. Short wavelength (UV)

illumination onto the gel from above, ensured good recovery of product from the gel. The band was excised from the gel and the oligonucleotide was eluted from the gel slice in 1 ml, TE at room temperature for at least 2 hours. Salt and acrylamide contaminants were removed by gel-filtration using "NAP-10" columns.

2.3.6 Radioactive Labelling of DNA sequences

Three methods were used to attach radioactive labels to DNA fragments and oligonucleotides: oligolabelling, Kinase end-labelling and terminal transferase end-labelling.

2.3.6.1 Oligolabelling

DNA fragments were oligolabelled to high specific activities using a method by Feinberg and Vogelstein (1984). Fragments to be labelled were either prepared as described in the previous section, or were labelled directly, still contained in low melting point agarose (1%). Prior to using these gel bound DNA fragments water was added to the samples at a ratio of 3 ml water/gram gel. The samples were then boiled for 10 min, in order to release the DNA from the gel matrix. The samples could at this point be stored in at -20°C . Prior to oligolabelling, the fragments were re-boiled for 3 min and were incubated at 37°C for 10 min. DNA fragments not embedded in agarose were boiled for 10 min and were transferred to ice, to be used immediately.

The labelling reaction was carried out using 10-100 ng DNA, 2 units of the large Klenow fragment of DNA polymerase I and 20-50 uCi ^{32}P alpha-dCTP (3000 Ci/mmol), in a final reaction volume of 50 ul. The reaction buffer contained 200mM N-2 hydroxyethyl piperazine

N'-2-ethane sulphonic acid (HEPES), pH.6.6, 50 mM Tris-HCl pH 7.5, 5 mM magnesium chloride, 10 mM beta-mercaptoethanol, 20 uM dATP, dGTP, dTTP, 5 O.D. units/ml hexadeoxyribonucleotides p(dN)₆ and 400 ug/ml BSA. The reaction was left to incubate at 19°C for a minimum 2 hours. Unincorporated nucleotide triphosphates were separated from the final product by gel filtration using Sephadex or using a "Nensorb-20" purification cartridge.

Oligonucleotides were labelled using essentially the same methods as above. In place of random hexanucleotide primers however specific complementary sixmer primers (0.2 uM) to the 3' end of the template oligonucleotide (0.06 uM) were used. Incubation was carried out at a temperature of 4°C to ensure hybridisation of the primers. To facilitate separation of the template DNA from the labelled product a 5' ³²P end-label was attached by Kinase labelling.

The reaction products could be separated by gel electrophoresis on a 20% polyacrylamide gel. Through short exposure to X-ray film the labelled oligonucleotide could be located and excised from the gel. The final product was again purified by gel filtration or using a "Nensorb-20" purification cartridge.

2.3.6.2 Kinase End-labelling

DNA was 5' end-labelled using T4 polynucleotide Kinase. For non-synthetic DNA to be end-labelled the 5' phosphate group had to first be removed. The enzyme calf alkaline phosphatase (CAP) dephosphorylated 0.5 ug DNA in the presence of 50 mM Tris-HCl, pH.9.0, 1mM magnesium chloride, 100mM zinc chloride, 1mM spermidine when added to a total volume of 100ul and incubated at 37°C. One unit of enzyme was added initially followed by another unit after 30 min. The enzyme was arrested after a further 30 min by incubation with 25 mM EDTA for

20 min at 65°C. The dephosphorylated DNA was extracted by phenol/chloroform extraction, which was followed by ethanol precipitation.

Kinase end-labelling was performed using the precipitate resuspended in 50 µl 50 mM Tris-HCl, pH 7.5, 10 mM magnesium chloride, 10 mM beta-mercaptoethanol, 25-50 µCi 32 P gamma-dATP (3000 or 7000 Ci/mmol) and 2 units of T4 polynucleotide kinase. The reaction was left to incubate at 37°C for a minimum of 15 min. and was then chased with 1 µM dATP and left for a further 15 min.

Oligonucleotides were end-labelled by a similar reaction. Because oligonucleotides are synthesised without the 5' phosphate group, dephosphorylation was not required. Generally 60 ng (10 pmol) of oligonucleotide material was labelled with 1-10 units of T4 polynucleotide Kinase enzyme and 30 µCi of 32 P gamma-dATP in a total volume of 10 µl.

To increase the proportion of labelled oligonucleotide in the final product the reaction mix could be resolved on a 20% polyacrylamide gel. Short exposure of the gel to X-ray film (X-Omat XAR-5 X-ray film) located the end-labelled oligonucleotide. Through careful excision from the gel (unlabelled oligonucleotide migrated slower than the labelled oligonucleotide) and elution in TE buffer high yields of labelled product could be recovered.

Unincorporated nucleotides and gel contaminants were removed either by gel filtration or by using a "Nensorb-20" purification cartridge.

2.3.6.3 Terminal Transferase End-labelling

Oligonucleotides were end-labelled using calf thymus terminal transferase in a restricted labelling reaction. This reaction

added a short homopolymer tail on to the 3' end of the oligonucleotide sequence. The manufacturers buffer was used (200mM potassium cacodylate, pH 7.0, 0.2 mM dithiothreitol, 1.5 mM cobalt chloride) in a reaction volume of 20 μ l along with 60 ng (10 μ mol) oligonucleotide, 30-50 μ Ci 32 P alpha-dCTP (3.32 μ mol/10 μ Ci 32 P dCTP) and 2 units of terminal transferase. The reaction was incubated for approximately 30 min. at 37°C. Unincorporated nucleotides were removed either by gel filtration or by purification through a "Nensorb-20" purification cartridge.

To calculate the efficiency of end-labelling and how many residues had been added per end, aliquots of the reaction mix were spotted onto DE-81 filter discs. The discs were washed and the radioactivity was counted by Cerenkov counting.

To increase homopolymeric tailing of the oligonucleotide, the concentration of radioactively labelled nucleotide added to the reaction was increased.

2.3.7 Non-radioactive Labelling of Oligonucleotides

Globin and sickle 20mer oligonucleotides were derivatised at the 5'-terminus during synthesis with an aminohexylphosphate linker (aminolink II, Applied Biosystems). The linker, a methyl phosphoramidite, was activated by tetrazole for the coupling reaction in a manner analogous to a normal nucleoside phosphoramidite. Normal cleavage and deprotection was carried out with ammonia. The alkylamine containing oligonucleotides were modified with biotin and with horse radish peroxidase enzyme (HRP).

2.3.7.1. Biotin Derivatisation

Oligonucleotides were biotinylated according to the methods described by Urdea (1988) Alkylamino oligonucleotides (3 OD 260 units) were taken up in 100ul of 0.1 sodium phosphate, pH.7.5 to which 100 ul of DMF containing 1 mg of "long chain" N-hydroxysuccinimidyl biotin was added. After 18 h at room temperature one tenth of the mixture (10 ug) was purified either through a "Nensorb-20" purification cartridge, or using a 20% polyacrylamide gel. Gel electrophoresis was performed over several hours (preferably overnight) and the purified product (a band below the unlabelled sequences) was eluted from the gel slice in 1ml TE. The purified and crude product were both stored at -20°C .

2.3.7.2 HRP Derivatisation

Horse radish peroxidase (HRP) labelled oligonucleotide probes were made according to Urdea (1988). To 3 OD 260 units lyophilised alkylamino oligonucleotide 25 ul of 0.1M sodium borate, pH 9.3 and 500 ul of distilled DMF containing 20 mg of p-phenylene diisothiocyanate (DITC) were added. The solution was vortexed and incubated for 2 h at room temperature in the dark. The reaction was vortexed again with 3 ml n-butanol and 3 ml water. The mixture was centrifuged (5000 rpm) and the yellowish upper layer discarded. This extraction process was repeated several times until the final volume remaining was approximately 50 ul. Any remaining butanol was removed by evacuation. Then 10 mg HRP in 200 ul of 0.1 M sodium borate, pH.9.3 was added. The mixture was vortexed and left over night at room temperature in the dark.

The HRP-DNA conjugate was separated from free enzyme and

oligonucleotide on a 7% polyacrylamide gel. The gel was run under standard conditions, allowing the dye front to reach 2/3 down the gel. The orange-brown band was cut out and eluted in 1 ml 0.1 sodium phosphate, pH.7.5 overnight at room temperature in the dark. Remaining contaminants and enzyme were either removed by purification through a "Nensorb-20" purification cartridge, or by filtration using a Centricon 10 Microconcentrator (Amicon). The HRP-DNA was concentrated by centrifugation at 3500 rpm and was washed twice with TE buffer. The final product was stored at -20°C.

2.3.8 General Methods:

2.3.8.1 Gel Filtration of Labelled Nucleic Acids

Unincorporated nucleotides were removed from labelling reactions either through using a Nensorb affinity column or by gel filtration. The column type used for gel filtration was DNA length dependent. Table 6. illustrates the properties of different purification methods. Each procedure was tested for its efficiency in separating unincorporated radioisotope from the labelled DNA product.

Pre-packed "Nap-10" columns and self prepared G-25 Sephadex columns were ideally suited for small fragments of DNA (>100 bp) and oligonucleotides. Sephadex G-50 was used for the gel filtration of longer DNA fragments.

The Sephadex columns were prepared either as drip columns or as spun columns in 1 ml syringes. For both columns the Sephadex used was equilibrated with TE buffer. Spun columns were prepared by packing the gel matrix into syringes, pre-blocked with siliconised (sigmacoted) glasswool plugs. Gel packing was achieved by centrifugation for 5 min at 3000 rpm. The columns were washed twice with 100 ul 20% ethanol

through centrifugation, using the same rotor speed and time. Sample (made up to 100ul volume with 20% ethanol) was then applied to the columns and the filtrate was collected by a further centrifugation step using the same conditions.

Drip columns were prepared either in 1 ml syringes or in siliconised Pasteur pipettes, blocked at one end with a siliconised glasswool plugs. The gel matrix was packed under gravity and was washed twice with 20% ethanol before applying the sample (>50 ul) to be purified. The filtrate was washed through the column with 20% ethanol and 100 ul fractions were collected. The first excluded peak of material, the presence of which could be detected by a change in absorbance measurements at O.D. 260 nm, was the purified probe.

Nap-10 columns were suited for gel filtrations where the sample volume was greater than 100 ul. They were used as drip columns from which 100 ul fractions were collected using 20% ethanol as a washing buffer. The concentration of purified probe was estimated from absorbance measurements of the elutant at O.D. 260 nm.

Gel filtration with Sephadex resins provided the cleanest separation of all nucleic acid probes, from unincorporated nucleotides. However, a more reliable method empirically was found to be purification with "Nensorb-20" columns. Using the protocol recommended by the manufacturers unincorporated nucleotides could be removed with almost 100% efficiency from the labelled DNA. The underlying principle of purification using these columns is that of ion-exchange chromatography. Associated with the purification of oligonucleotides was a 20-30% loss of probe, which occurred when short sequences (>100bp) were applied (table 6).

Non-radioactively end-labelled DNAs could also be purified using "Nensorb-20" affinity columns. This method suited the extraction of non-radioactively labelled products as there was no need to

determine colorimetrically when the labelled product was completely removed.

2.3.8.2 Determination of Radioactivity

³²P-Radioactivity was measured by Cerenkov counting at an efficiency of approximately 25% or by scintillation counting in a water-based scintillant Aquasol. The measurements were made using an LKB 121 4 Rack beta liquid scintillation counter.

2.3.9 Application of DNA to Membranes for Hybridisation

Genomic and recombinant DNA fragments were applied to nitrocellulose or nylon filters either using the direct methods of slot/dot-blotting or by indirect transfer through Southern blotting.

2.3.9.1 Slot/Dot Blotting

Starting with purified samples of restricted DNA fragments, the DNA was denatured in one of two ways: 1) either by heat denaturation at 98°C for 10 min followed by snap freezing in a dry cardice bath; or 2) by adding 0.1 volume of 3M NaOH to a final concentration of 0.3-0.4 M and incubating this solution for 1 h at 65°C. The solution was then cooled to room temperature and 1.0 volume of 2 M ammonium acetate, pH.7.0 to a final concentration of 1 M was added.

The single stranded DNA was applied to nitrocellulose or nylon filters by direct dot blotting using a Gilson pipette or under vacuum using a "slot-blot" apparatus (Hybaid) The DNA was irreversibly bound to the filter by baking in an oven at 80°C for 2-16 h.

2.3.9.2 Southern Transfer

Enzyme digested or PCR amplified DNA fragments for transfer were size fractionated through gel electrophoresis along with Kinase end-labelled size markers. The markers used were either radioactively labelled, Hind III digested lambda DNA, or Hae III digested X174. After electrophoresis was completed the gels were stained with ethidium bromide and photographed, using FP4 film. The gels were then denatured for 45 min in 0.5 M NaOH, 1.5 M NaCl with gentle shaking at room temperature. This was followed by neutralisation in 0.5 M Tris HCl, pH.7.5, 1.5 M NaCl for 45 min also at room temperature. The DNA was transferred to nitrocellulose filter by capillary action, as described by Southern (1975). Blotting was carried out overnight (16h). The filter was then washed briefly in distilled water, blotted dry and was baked in a Whatman 3MM envelope for a minimum of 2 h (maximum 16 h) at 80°C.

2.3.10 Membrane Hybridisations

Nitrocellulose and nylon filters with non-covalently bound DNA, were prehybridised in plastic hybridisation bags, for a minimum of 1 h, in 6 x NET, 0.1% SDS, 3% BSA, 0.5 mg/ml sonicated, denatured salmon sperm DNA or tRNA, and approximately 0.02 mg/ml non-specific 10mer oligonucleotide. Hybridisations were carried out in a fresh solution of the same buffer using 10-20 mls buffer per 100-200 cm filter.

Denatured, labelled probe DNA was added to the hybridisation mix and the bags were incubated (with shaking) in sandwich boxes containing water, overnight. The hybridisation and prehybridisation temperatures were optimised for the probe being used. Oligonucleotides were generally hybridised 10°C below the calculated melting

temperature whereas longer probes (<30 bp) were hybridised at 65°C.

After incubation the filters were removed and washed three times in cold (4°C) 6 x NET, 0.1% SDS. For long recombinant DNA probes the filters were then washed in 1 x NET, 0.1% SDS, for two 30 min washes at 65°C (with gentle shaking). For oligonucleotide probes the filters were washed in 4 x NET, 0.1% SDS, for one 10-15 min wash at 50°C (with gentle shaking).

Differential hybridisation between oligonucleotide pairs with single base differences was achieved by changing the stringency of further washing manipulations. A 10 min wash using the same washing solution, at the melting temperature of the mismatched oligonucleotide, resulted in dissociation of the mismatched hybrid duplexes, with target DNA. Matched duplexes were not affected. The filters were then blotted dry, covered in clingfilm and transferred to X-ray cassettes (with intensifying screens) for autoradiography with X-Omat XAR-5 X-ray film (Kodak).

2.3.11 Direct Gel Hybridisations with Oligonucleotide Probes

Gels for direct hybridisation with oligonucleotide probes were pre-treated in the same way as filters for Southern or dot-blotting. Once neutralised the gel was placed on two sheets of Whatman 3MM paper and covered with clingfilm. The gel was then transferred to a gel dryer (Hoeffer Scientific Instruments) and left to dry, initially without heat. When almost dry (flat), the heater was set to 60°C and the gel was left for a further 30 min. The gel could then be stored indefinitely at 4°C, or until needed.

Gel hybridisations were performed using the same protocol as for filter hybridisations. Pre-hybridisation of the agarose gel was however not required. Prior to hybridisation the dried gel was removed

from the paper backing, by floating it on a shallow pan of water for a few minutes. The paper could then be peeled away from the gel. Unlike nitrocellulose, a dried agarose gel could be repeatedly subjected to washing manipulations following the initial wash and exposure to autoradiography. This property permitted gels to be subjected to repeated stringent washes, until the optimum signal-to-noise ratio was obtained.

2.3.12 General Methods:

2.3.12.1 Autoradiography

Filters and dried gels were wrapped in clingfilm and autoradiographed between two intensifying screens at -70°C . The film used, X-OMAT XAR-5 X-ray film was desensitized by pre-flashing to an O.D. 540 nm of 0.1-0.2.

Polyacrylamide gels used to locate radioactively labelled product for extraction, were covered with clingfilm and exposed to X-ray film directly for 15-120 seconds. For this only one glass electrophoresis plate was removed. Small incisions were made through the film and gel, using a scalpel blade in order to locate the labelled product accurately.

2.3.12.2 Densitometric Scanning of Autoradiographs

The intensity of signals and bands on autoradiographs were evaluated using a Chromoscan (Joyce Lobel Chromoscan) densitometer.

2.3.13 Detection of Non-radioactively Labelled Probes

Oligonucleotides labelled with biotin or HRP were detected colorimetrically using both soluble and insoluble substrates for resin based and filter hybridisations respectively.

Biotin moieties (10 pmol) were reacted with a streptavidine horseradish peroxidase conjugate (SA-HRP) in concentrations recommended by the manufacturer (1ul of the stock solution). Following normal hybridisation and washing procedures the conjugate was added in 10ml of fresh buffer, 6 x NET, 0.1% SDS, 0.3mg/ml poly A. The reaction was left for 1 hour with gentle shaking, at 37°C in the dark. The beaded supports or filters were then washed twice with 4x NET, 0.1% SDS and were stored in this buffer until assayed colorimetrically for HRP.

2.3.13.1 Filter Detection

For colorimetric detection of HRP, filters were incubated with 10 ml of 0.1 M Tris-HCl, pH 7.4, 2 ml of ethanol, 6 mg of 3,3'-diansidine, 10 mM imidazole, and 6 ul of 30 % hydrogen peroxide. The filters were developed in the dark at room temperature and the reaction was terminated by washing the filters with distilled water. The filters were blotted dry and could be stored in an envelope of Whatman 3 MM at room temperature.

Detection of the labelled oligonucleotides was also carried out as described by Sheldon (1986). The hybridised filters were first incubated for 5 min at room temperature in 100 ml of phosphate-buffered saline containing 100 mM sodium chloride, 1 M urea, 5 % Triton X-100, and 1% dextran sulphate, then equilibrated in 100 mM sodium citrate pH 5.0, containing 0.1 mg of 3,3',5,5'-tetramethylbenzidine per ml. Colour development was achieved by transferring the filters to

sodium citrate-tetramethylbenzidine buffer containing 0.0015% hydrogen peroxide, and then incubating them for 10-20 min at room temperature. The filters were washed in distilled water to stop the reaction and stored as above.

2.3.13.2 Bead Assay Detection

For hybridisation reactions with immobilised oligonucleotide probe and using HRP end-labelled detector probe the following assay was performed to confirm the presence of hybridised target DNA (sandwich hybridisation assay). To each reaction vessel, 100ul aliquot of a fresh o-phenylenediamine solution, 50 mg OPD dissolved in 5 ml of 50 mM sodium citrate, pH.5.1, containing 3 ul of 30 % hydrogen peroxide was added. After 20 min at 37°C, 50 ul of 4N sulfuric acid was added to quench the reaction. The beads were then pelleted by centrifugation and the supernatant was transferred to a microtitre dish well. The dish was read on a Labsystems Multiscan MCC plate reader set at O.D.490 nm.

2.3.14 Immobilisation of DNA to Beaded Supports

DNA fragments and oligonucleotides were covalently immobilised to solid beaded supports using three methods: 1) by carbodiimide activation, 2) enzymically and 3) by diazotization.

2.3.14.1 Immobilisation of DNA by Carbodiimide Activation

The immobilisation of DNA by carbodiimide activation was carried out essentially according to Allfrey and Inoue (1978). DNA fragments and oligonucleotides were attached by their 5' ends using

1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate (carbodiimide or CMC). The method relied on the availability of the 5' phosphate group to react with the hydroxyl groups on the support resin. Oligonucleotides therefore required kinase end-labelling of the 5' ends with phosphate groups prior to immobilisation.

The DNA (25 pmol) was denatured by boiling in 100 ul 40 mM Na MES buffer, pH.6.0 and cooled in dry carbondioxide/ethanol (cardice bath). The DNA samples were then added to 100 mg aliquots of resin which had been pre-washed in 40 mM Na MES buffer, pH.6.0. The samples were dried for 2 hours in a speed Vac concentrator (Savant) prior to activation with carbodiimide.

Activation was initiated by the addition of 28 ul of 80 mg carbodiimide/ml distilled water to the dry resin. The sample was boiled for 7 min and then rapidly cooled in a cardice-bath. A second aliquot of carbodiimide was added this time dissolved in 40 mM MES pH.6.0. The sample was boiled for another 7 min and cooled in the cardice bath. Lastly, 140 ul of 0.1 M NaCl, 1mM EDTA, 10 mM sodium phosphate pH.7.0 (NEP buffer) was added and the sample was left to swell overnight at 4°C.

The supernate was discarded and the resin resuspended in 4 ml freshly made 0.2 M sodium carbonate buffer, pH.10.2. The samples were washed overnight at 4°C, with end over end mixing on a rotating (Voss) wheel. This step was important to denature any nonspecific bonds formed between the resin and the bases of the DNA. Finally the resin was washed six times with 1 ml NEP buffer and stored in TE at 4°C.

2.3.14.2 Enzymic Immobilisation

A method developed by Goldkorn (1986) for the attachment of DNA fragments to cellulose was adapted for the immobilisation of

oligonucleotides. This method relied on the activities of ligase and Klenow enzyme to covalently link the oligonucleotide to oligo (dN) cellulose.

A pair of complementary oligonucleotides were dried under vacuum using a speed vac concentrator (Savant). One oligonucleotide was synthesised with a 15 base (dA) homopolymer tail. This oligonucleotide (35mer) served as a "linker" to the other sequence (20mer or 50mer) phosphorylated at the 5' end, to be immobilised to the solid support.

The oligonucleotides were resuspended in 200 μ l, 0.5 M NaCl, 10 mM Tris HCl pH 7.0 (high salt buffer) and denatured in a boiling water bath for 10 min. The oligonucleotides were then added to 1 mg oligo (dT or dU) cellulose and incubated at 50°C for 30 min, with gentle shaking. The suspension was left to cool to room temperature for another 30 min. The resin was subsequently washed two times in 200 μ l of the high salt buffer, pelleting the resin at slow speeds (6500 rpm) in a microfuge and decanting the supernate after each wash. Two further washes were carried out using 200 μ l, 0.1 M NaCl and 10 mM Tris HCl, pH 7.5 (low salt buffer).

After the fourth wash the supernate was discarded and 50 μ l Klenow buffer (80 μ M dATP, dCTP, dGTP, dTTP, 0.1 M NaCl, 10 mM magnesium sulphate, 0.1 mM DTT, 0.05 mg/1 BSA, 50 mM Tris HCl pH 7.2) was added together with 1 μ l (2 units) Klenow enzyme. The suspension was incubated at 37°C for 30 min.

A ligation buffer (5 mM magnesium chloride, 5 mM DTT, 0.1 M ATP 70 mM Tris HCl pH 7.6), plus an additional 7.5 mM magnesium chloride and 10-20 units T4 DNA ligase were added to the reaction, bringing the final volume to 200 μ l. The ligation reaction could also be carried out using ligase buffers supplied by the manufacturer. The reaction was left at 25°C for a minimum of 30 min (preferably

overnight).

Following this the resin was washed two times with both the high and low salt buffers respectively. Finally the resin was heat treated at 98°C for 5 min. in a boiling waterbath, and washed a further two times in low salt buffer. The final product was stored in low salt buffer at 4°C.

2.3.14.3 Immobilisation of DNA by Diazotization

DNA fragments and oligonucleotides were immobilised by diazotization according to a reaction described by Seed (1982) and using a protocol by Bunemann (1982). Aliquots of resin (20 g) were washed with distilled water and were transferred to a sterile 50 ml Falcon tube. 20 ml 1 M NaOH and 1.7 ml 1,4-butanediol-diglycidyl ether were added and the slurry was mixed end over end on a rotating wheel (Voss) at room temperature overnight.

The mixture was then transferred to a Buchner funnel and the aqueous solution removed completely. This was achieved by pressing out the solution with a spatula until only a moist cake was left. The resin was placed back into the Falcon tube to which 23 ml acetone and 2.86 ml 2-aminothiophenol were added. The slurry was again mixed end over end at room temperature overnight.

Finally the resin was washed in a Buchner funnel (in the fume hood) with 200 ml acetone followed by washes in 0.1 M HCl and water, 0.1 M HCl and water again. The resulting 2-aminophenylthioester (APTE) derivative of the resin was suspended in water and stored at 4°C.

Aliquots of the APTE resin were diazotized by first washing the resin in 30 ml distilled water through a sintered glass filter. The resin was then resuspended in 1 ml distilled water, 3.33 ml 1.8 M HCl and kept on ice for 30 min. The activated resin was reacted with

successive additions of 33 μ l aliquots of freshly made up sodium nitrite 10 mg/ml. The additions were made in 7 min intervals and after each the resin was tested with starch iodide paper. The reaction was completed when the indicator paper remained positive (dark blue). This usually occurred after 4 additions of sodium nitrite. The slurry was then quickly transferred into an ice-cold sintered glass filter and washed immediately with 50 ml ice-cold water.

Finally the mixture was washed with 5 ml ice-cold 25 mM sodium phosphate, pH.6.0 and dimethyl sulphoxide (DMSO) (20:80 v/v). The resin usually turned a darker colour during this last washing step. As soon as the buffer was drained away, 500mg of the wet resin was added to 250 μ l of DNA solution in a screw capped eppendorf.

The DNA solution was prepared by dissolving the dried DNA pellet in 50 μ l 25 mM sodium phosphate pH.8.0 and 200 μ l DMSO.

The reaction mixture was incubated for 2 days on a rotating wheel at room temperature in the dark. The appearance of small air bubbles in the mixture were a good indicator of a successful reaction. The suspension was then carefully washed with water followed by a wash in 0.4 M NaOH pre-warmed to 40°C. The resin was finally washed with 100 ml TE at room temperature and could then be stored in the same buffer at 4°C.

2.3.15 Quantitation of Immobilised DNA

The quantity of DNA covalently coupled to the resins was determined primarily by incorporating radioactive tracers into the reaction. For resins with DNA immobilised by carbodiimide activation and by diazotization an S1 nuclease digestion could also be carried out to establish the amount of DNA potentially available for hybridisation. Furthermore a mock hybridisation test could establish

the amount of covalently immobilised DNA lost through heat treatment or mechanical sheering.

2.3.15.1 Radiolabelling

A trace of 32 P-kinase labelled DNA (5% of the total) was incorporated into control immobilisation reactions. In another control reaction the DNA sequence was left unimmobilised and untreated. By comparing the initial counts of the control reactions through Cerenkov counting with the final counts, the percentage DNA specifically bound to the support could be determined. Final counts were made after the last washing manipulation, when all unbound DNA was completely removed, and initial counts were made at the start of the immobilisation reaction.

2.3.15.2 S 1 Nuclease Digestion

Nuclease digestions were carried out with aliquots of the radioactively labelled resin samples. 10-20 mg of resin samples were resuspended in 20 mM NaCl, 5 mM sodium acetate, 0.1 mM zinc sulphate and 5% glycerol. The enzyme S 1 nuclease was added to a final concentration of 500 units/ml, and the mixture was incubated at 37°C overnight. The resin were Cerenkov counted and then washed several times by centrifugation. This was done using a microfuge and substituting the reaction buffer for a fresh aliquot of TE buffer.

The amount of DNA available to S1 nuclease digestion was estimated from the Cerenkov counts of the pooled supernates, recovered after each wash. The radioactive counts lost were an indication of the amount of DNA covalently immobilised to the solid support and potentially available for hybridisation.

2.3.15.3 Determining the Stability of Immobilisation

Mock hybridisations with all of the appropriate hybridisation buffers and temperatures were performed to determine the stability of covalent immobilisation to each beaded support matrix. For these reactions 10-20mg of the beaded control samples (radioactively labelled with ^{32}P) were used. Each reaction was left for one hour. The resins were then washed several times by exchanging the buffer solution after precipitation of the resins through centrifugation (using a microfuge).

The radioactivity lost to the supernatant could be Cerenkov counted and was an indication of the chemical or mechanical leakage of immobilised DNA, occurring from the resin.

2.3.16 Hybridisations with Resin Immobilised DNA Probes

Hybridisations of Kinase end-labelled target DNAs to resin immobilised DNA probes were carried out as simple one-step reactions. Sandwich hybridisations, which involved the hybridisation of a third nucleic acid component, were performed either as one-step (a single reaction) or two-step assays (two reactions adding target and detector probe at different times). In these assays the target DNA remained unlabelled and the detector probe was end-labelled with either ^{32}P or HRP enzyme.

Hybridisations were performed in 1.5 ml sterile screw cap eppendorf tubes. The resins used, were prehybridised for 1 hour in a shaking waterbath and the following hybridisation buffer: 200ul, 6 x NET, 0.1% SDS, 3% BSA, 0.5 mg/ml sonicated, denatured salmon sperm DNA or tRNA, and approximately 0.02 mg/ml non-specific 10mer oligonucleotide. Following this the resins were pelleted in a

microfuge and the supernates were discarded.

The hybridisation of nucleic acid components, was carried out in 100ul or 50ul of the same buffer used for prehybridisation, with the appropriate free single-stranded target DNA.

Incubation temperatures for both prehybridisation and hybridisation were dependent on the sequence lengths of the different nucleic acid components involved in the assay. Temperatures were chosen to suit hybridisation of the shortest DNA fragment or oligonucleotide. Typically if a 20mer oligonucleotide was involved in the reaction, temperatures were kept to 50°C. Long DNA fragments on the other hand were prehybridised and hybridised at 65°C.

The hybridisation incubation time used was generally 16 hours although optimum times were found to suit the different reactions (see the results).

Post hybridisation washing manipulations were carried out in 6 x NET, 0.1 % SDS, first at room temperature and then at the hybridisation temperature. The washes were achieved by pelleting the resins for 30 seconds at low rpm (6500), removing the supernate and substituting the discarded buffer for a fresh aliquot of buffer. Generally three washes were required to remove all of the unhybridised labelled probe.

For differential oligonucleotide hybridisations the resin:hybrid complexes were subjected to further washes using the same stringent conditions as described in section 2.3.9.3.

Hybridisation efficiencies were established by comparing the initial with the final Cerenkov counts immobilised to the resin samples. The specificity of detector probe hybridisation on the other hand was determined by comparing the total resin counts with those obtained for non-specific hybridisations with non-complementary detector probes, or target DNAs.

For all of these hybridisations it was important to use labelled probes, purified from unincorporated label. This ensured that the signal immobilised to the resin upon hybridisation, would only represent specific hybridisations and non-specific bindings of the detector probe. For this purpose, purification using "Nensorb-20" purification cartridges was found to be most efficient.

RESULTS

3.1 Preparation of oligonucleotides and restriction fragments

This study was aimed at utilising the specific hybridisation characteristics of oligonucleotides to detect single base mutations within particular DNA sequences. Oligonucleotides directed at the sickle cell disease mutation found in the beta globin gene, and the Z and S alleles in the alpha-1-antitrypsin gene, formed the basis for a sandwich hybridisation assay (figure 18). This assay would distinguish between normal and mutant target genes, under stringent hybridisation conditions.

The assay involved one synthetic oligonucleotide, immobilised to a resin support, designated as the "capture probe". This probe would serve to attach the specific target DNA through hybridisation to a solid support. A second labelled oligonucleotide down-stream of the same sequence strand, the "detector probe", would hybridise to the target mutation site. The differential stability of exactly matched and mismatched hybrid duplexes would then enable the differentiation between normal and mutated target sequences, and thus establish the genotype.

For these purposes oligonucleotides were synthesised using either phosphite-triester or phosphotriester chemistries. To establish the optimum assay conditions for target DNA hybridisation to both these oligonucleotide probes, recombinant DNAs containing both the beta globin and the alpha-1-antitrypsin genes were used. The target DNAs were isolated from the vector DNA and were reduced to suitable lengths through restriction enzyme digestion. Hybridisations could then be set up to determine the required properties of each of these probes for the assay being developed.

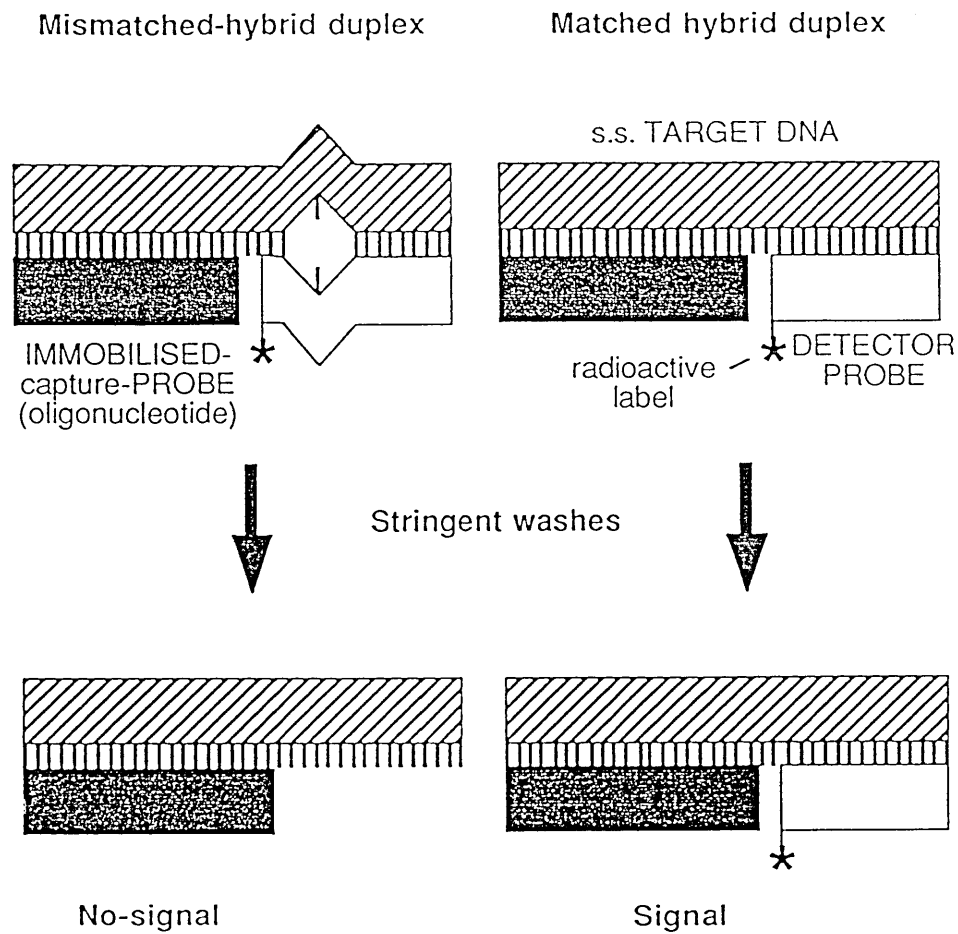


FIGURE 18 Differential Sandwich Hybridisation with 2 Oligonucleotide Probes

3.1.1 Oligonucleotide preparation

The two oligonucleotide probes to be incorporated into this sandwich hybridisation assay were chosen for different properties. The "capture probe" was designed to provide suitable reactive sites for immobilisation, leaving sufficient sequence available for stable hybrid formation with target DNAs. The "detector probe" was designed to provide the optimum sequence for detecting single base changes within the target DNA. The detector probe was designated such, as it was to carry the radioactive or non-radioactive label from which it would be recognised.

A variety of "capture probe" oligonucleotides were synthesised to find lengths that would suit the three coupling chemistries used and to find the minimum lengths required for stable hybrid formation with single-stranded target DNA's. The synthesised sequences coded for either the sense (s) or the anti-sense (as) DNA strands of the gene being analysed (table 7). It was decided that the minimum length of these probes would be 20 nucleotides long, as smaller lengths would not ensure sequence specificity upon hybridisation to human genomic DNA's.

A number of "detector probes" were synthesised, the sequences of which are listed in table 7. This specific hybridisation probe was generally used in pairs. One oligonucleotide was homologous to the normal gene sequence, whilst the other was homologous to the mutant gene sequence, at a region around the mutation site.

These probes were synthesised as 20mers to ensure sequence specificity upon hybridisation, and to enable differential hybridisation to the target DNA. For an oligonucleotide to hybridise to unique sequences of the human genome, a minimum length of 17 nucleotides is required. The maximum length for oligonucleotides used in this way is also limited by the melting temperature. Each sequence

Detector probes:

1. Alpha 1 -antitrypsin:

- a) GT CCC TTT CTC GTC GAT GGT (Z normal 20mer) s
- b) CTT (Z mutant 20mer) s
- c) GAG TTC ATT TTC CAG GAG CT (S normal 20mer) s
- d) TAC (S mutant 20mer) s

2. Beta Globin :

- a) CTG ACT CCT GAG GAG AAG TC (beta normal 20mer) as
- b) GTG (beta sickle 20mer) as
- c) GAC TGA GGA CTC CTC TTC AG (beta normal 20mer) s

3. 2-aminoadenine containing beta globin:

- a) CTG ACT CCT GXG GAG AAG TC (X = 2 aminoadenine analogue) as

4. PCO 4 beta globin:

- a) CAA CTT CAT CCA CGT TCA CC

(complementary to the beta globin gene up-stream from the sickle mutation site) s

.....

Capture probes: (Sequences coding for regions of the beta globin gene)

- a) GAC TGA GGA CTC CTC TTC AG (beta normal 20mer) s
- c) CTG ACT CCT GAG GAG AAG TCA AAA AAA AAA AAA AA (beta normal 20mer with a 3'dA tail) as
- d) GACTTCTCCT CAGGAGTCAG GTGCACCATG GTGTCTCTTT GAGGTTGCTA (beta normal 50mer, with extended 3'end) s
- e) AGGGCTGGGC ATAAAAGTCA GGGCAGAGCC ATCTATTGCT TAGATTGCT TCTGACACAA CTGTGTTTAC TAGCAACCTC AAACAGACAC CATGGTGCAC

(100mer sequence upstream from, but adjacent to the 20mer beta globin probe) as

Note: s = sense strand, as = anti-sense strand

TABLE 7 OLIGONUCLEOTIDES SYNTHESISED FOR THE DETECTION OF TARGET DNA AND SINGLE BASE CHANGES

requires a melting temperature which can be accurately determined. For this reason oligonucleotides were limited in length so that their melting temperature would be below 65°C.

In order to maximise the difference in melting temperature between the two detector probes, the nucleotides involved in the base mismatch were optimised for maximum instability, wherever possible. Oligonucleotide sequences coding for the normal gene were therefore chosen to contain cytosine or adenine residues at the point of mutation. This was to ensure maximum hybrid instability when hybridised to the abnormal target DNA.

Fundamental to this sandwich-hybridisation assay was the uniform length and composition of samples of capture or detector probes. It was therefore essential to carry out suitable post-synthetic purification to remove all incomplete sequences. Three methods were examined: 1.) acrylamide gel purification, 2.) ion-exchange chromatography using FPLC or HPLC, and 3.) purification using a commercially available affinity (Nensorb prep) column. The most reliable and efficient procedure was found to be purification using fast protein liquid chromatography (FPLC). This technique not only confirmed size of the purified product, but was also rapid and resulted in high yields. A comparison between the three techniques is depicted in table 8. FPLC purification was particularly suited for the longer capture probes (ie. 50mer and 100mer) where synthetic yields were low. Typical results for the characterisation and purification of a 20mer oligonucleotide using acrylamide gel procedures, by FPLC and an HPLC can be seen in figures 19, 20, 21.

	<u>Acrylamide</u>	<u>HPLC/FPLC</u>	<u>Nensorb</u>
<u>maximum loading capacity:</u>	1/2 of total synthesis	20-200 ug (1-2 OD 254nm)	total synthesis
<u>% recovery:</u>	40%	+ 90%	40%
<u>% purity:</u>	contaminating 19mers	100%	100%
<u>time required:</u>	4 hrs	20 min.-1 h	+ 1 hr

**TABLE 8 A COMPARISON OF THREE METHODS USED FOR
THE POST SYNTHETIC PURIFICATION OF
OLIGONUCLEOTIDES.**

The estimates are based on the purification of crude 20mer oligonucleotide, already cleaved from the solid support used during synthesis.

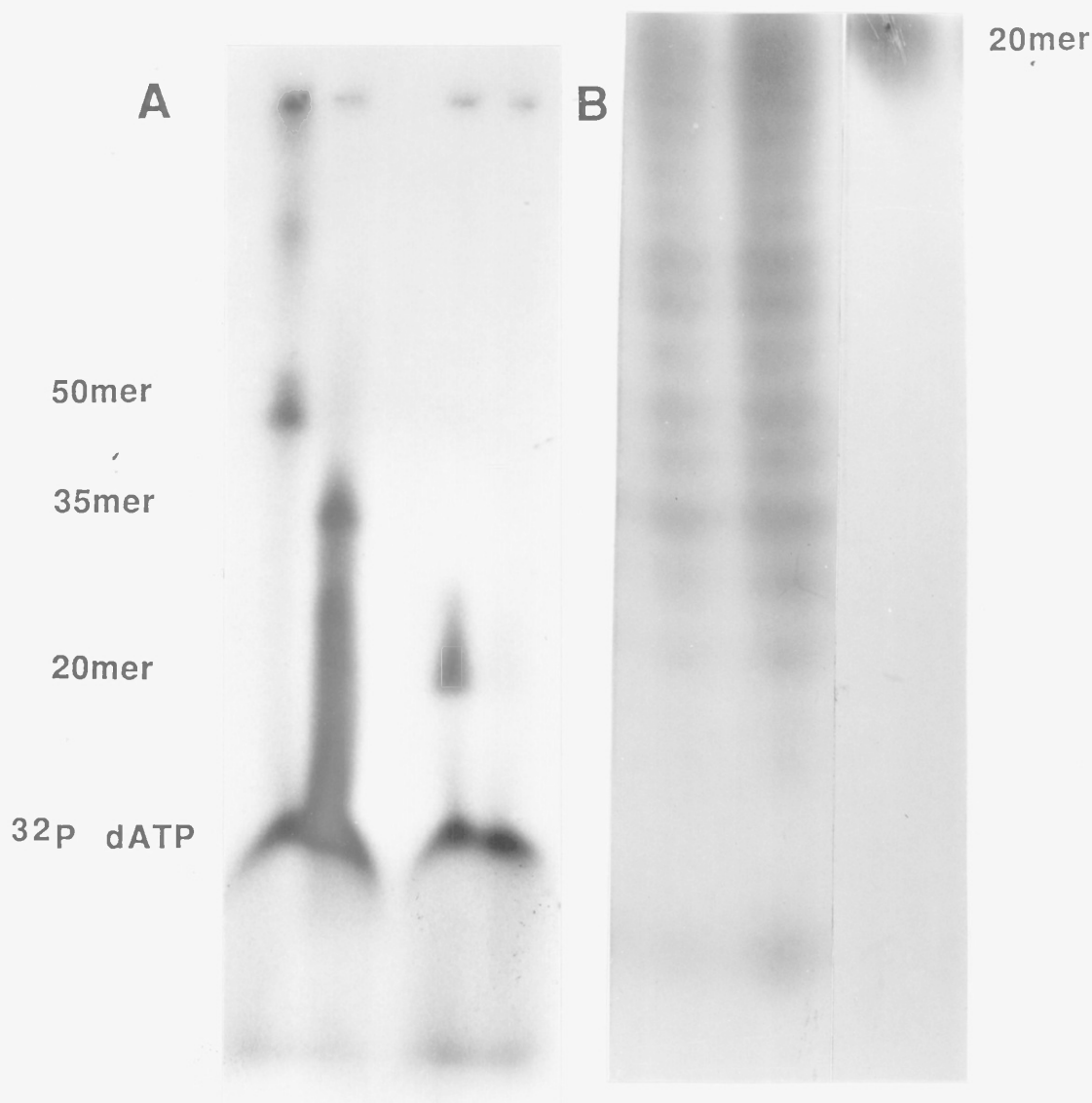


FIGURE 19 POLYACRYLAMIDE GEL ELECTROPHORESIS FOR THE CHARACTERISATION AND PURIFICATION OF SYNTHETIC OLIGONUCLEOTIDES.

A Lane 1. and 3. contain FPLC. purified 50mer and 20mer (1 pmol.) respectively. Lane 2. contains crude, unpurified 35mer oligonucleotide synthesised by the phosphotriester method on an automatic oligonucleotide synthesiser (1ul of total synthesis). All three oligonucleotides are ^{32}P kinase end-labelled and have been resolved on a 15% urea polyacrylamide gel. Autoradiography was for three minutes, exposing the film (X-OMAT X-AR 5) to the cling film covered gel directly. Low molecular weight contaminants are clearly removed through FPLC purification.

B Lanes 1. and 2. contain crude 20mer oligonucleotide synthesised by , manual phosphitetriester synthesis. Lane 3 contains acrylamide gel purified 20mer oligonucleotide. All three oligonucleotide samples have been ^{32}P kinase end-labelled and have been resolved on a 20% urea polyacrylamide gel. Using a high % acrylamide gel, small contaminants are clearly resolved. The darker bands in lanes 1. and 2. reflect the stages at which low coupling yields were seen during synthesis. The results also show the contamination of 19mers in lane 3, which are unavoidable when acrylamide purification strategies are used.

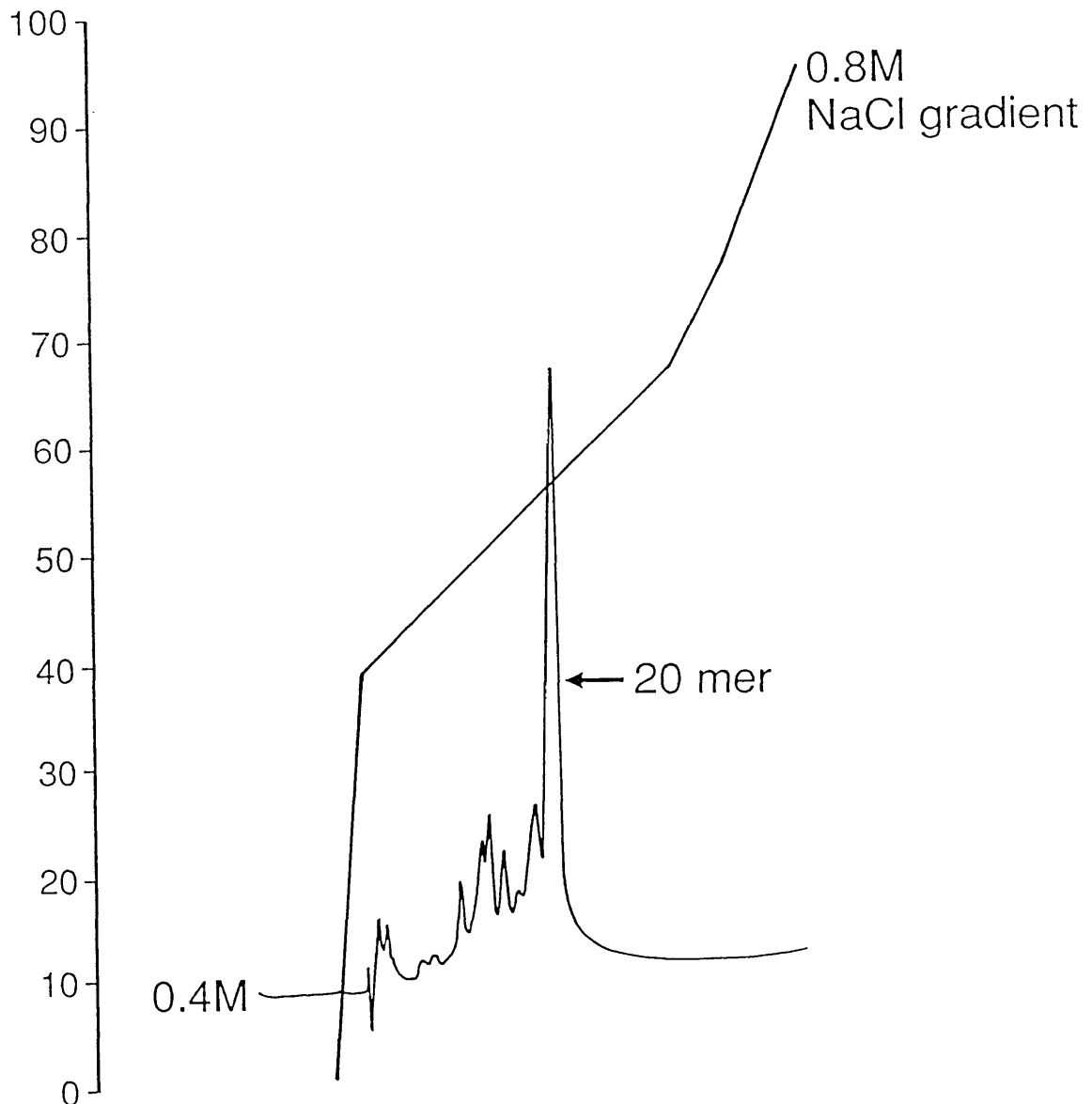


FIGURE 20

The purification of a 20mer Oligonucleotide using FPLC and an ion exchange column.

The technique relies on ion exchange chromatography using a Mono Q HR 5/5 (Pharmacia Ltd.) column. Using a flow rate of 2mls/min a salt gradient was set up, increasing from low (0.4 M NaCl) to high (0.8 M NaCl) salt concentrations in 20 minutes. The results show a clean separation of 20mer product from a crude mixture of oligomers in under 20 minutes. The maximum loading capacity for this clean separation is 20-50ug unpurified oligonucleotide (2 O.D. 254).

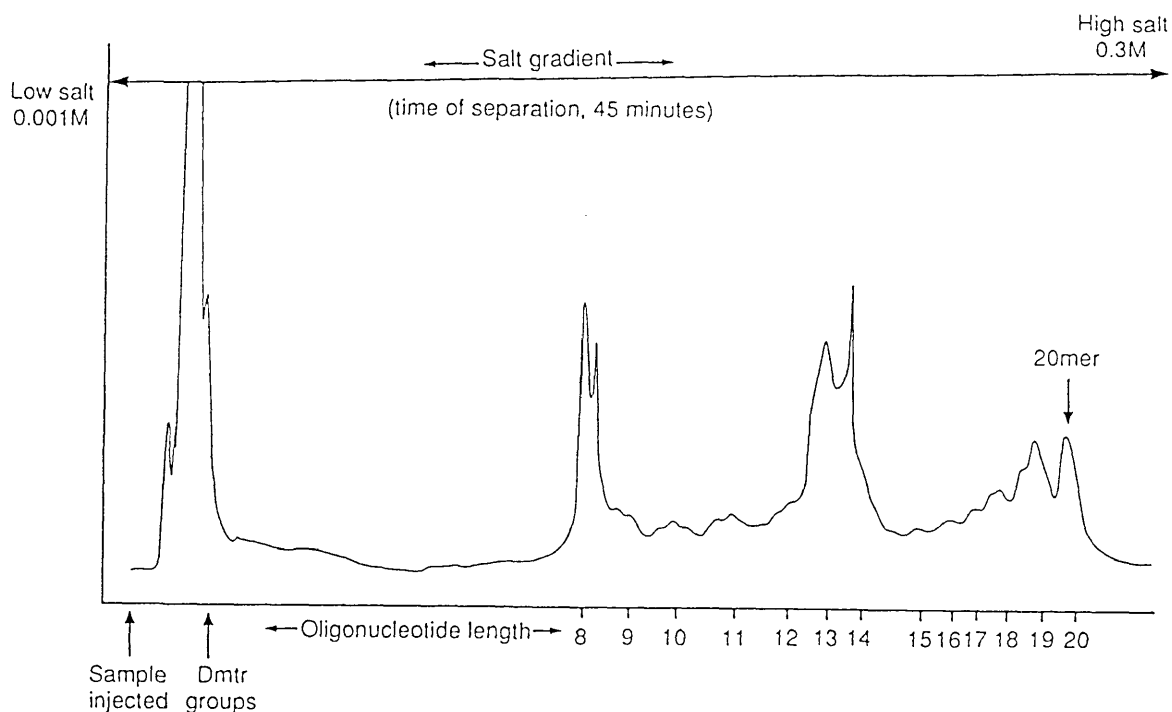


FIGURE 21

Purification of a 20mer oligonucleotide using HPLC

Purification of the synthetic product was performed using a Whatman Partisil 10-SAX ion-exchange column. A salt gradient was set up using 0.001 M and 0.3 M phosphate buffer, in the presence of 60% formamide and using a flow rate of 2.5 mls/min, over a period of 40 min. Purified 20mer oligonucleotide could be retrieved after 45 minutes. The maximum loading capacity for clean separation, was found to be 200ug (20 O.D.254).

3.1.2 Preparation of restriction fragments

A number of different restriction fragments were prepared from recombinant plasmid DNAs for use as target DNA in preliminary hybridisation experiments. The gene inserts for both the normal and mutant beta-globin and alpha-1-antitrypsin genes were excised from the vector DNA through restriction enzyme digestion with either Bam HI or Pst I. Restriction maps of the human DNA sequence inserts for both genes are shown in figures 22. and 23.

A proportion of the beta globin gene inserts were further purified and isolated from the vector DNA by electroeluting the fragments from agarose gel slices, upon electrophoresis. These 1900 kbp inserts eventually served as probes for one-step hybridisations, to assess the efficiency of the immobilised capture probe. Using the "Biotrap" gel apparatus an average recovery of 80-90% crude product was obtained. After two further desalting purifications, using "Nensorb-20" columns, a final recovery of between 40-50% was found.

The possibility of using FPLC as a method of extracting DNA inserts from vectors was examined. Large concentrations of material were however required to resolve the fragments. The final yields were between 80-90%, which after desalting gave a recovery between 60-70%. A typical FPLC profile can be seen in figure 24.

The 1900 kbp beta-globin inserts were also restriction digested with the enzyme Hae III, which generated a 208bp fragment around the mutation site. Six fragments were produced with this digestion: 209, 1045, 131, **208**, 272 and 66bp. The locations of four of the restriction sites are depicted in figure 22. The 208bp fragment, containing the sickle site, was chosen to determine the effect target length had on the stability of a sandwich-hybridisation complex. No attempt was made to isolate this fragment from the remaining five, as they were all

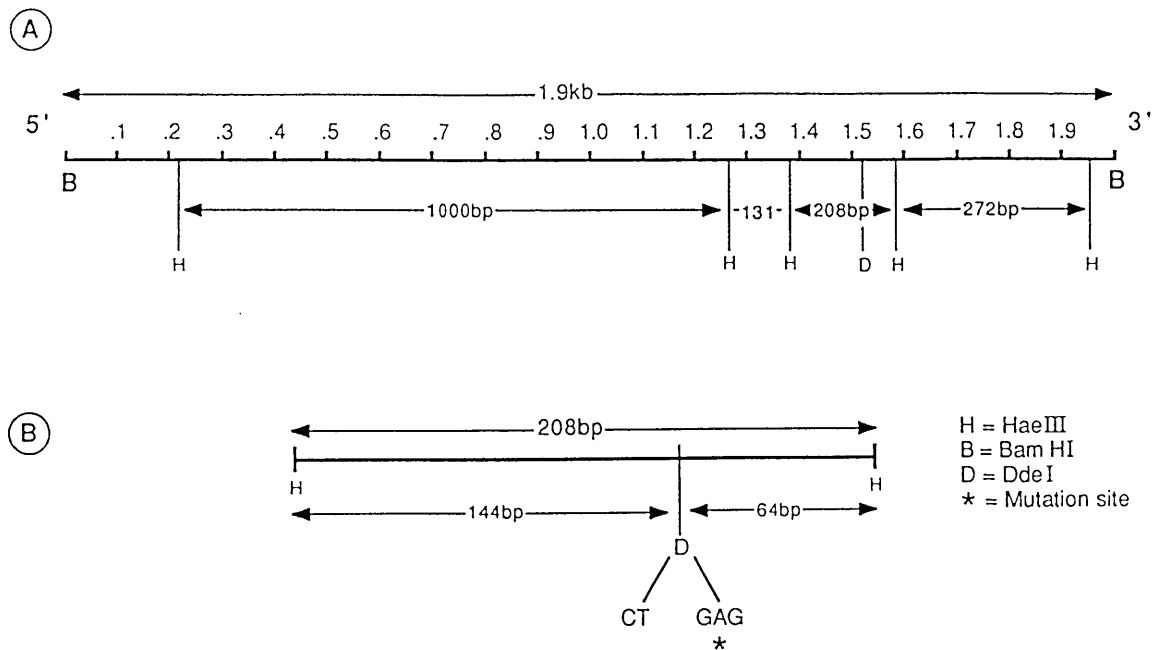
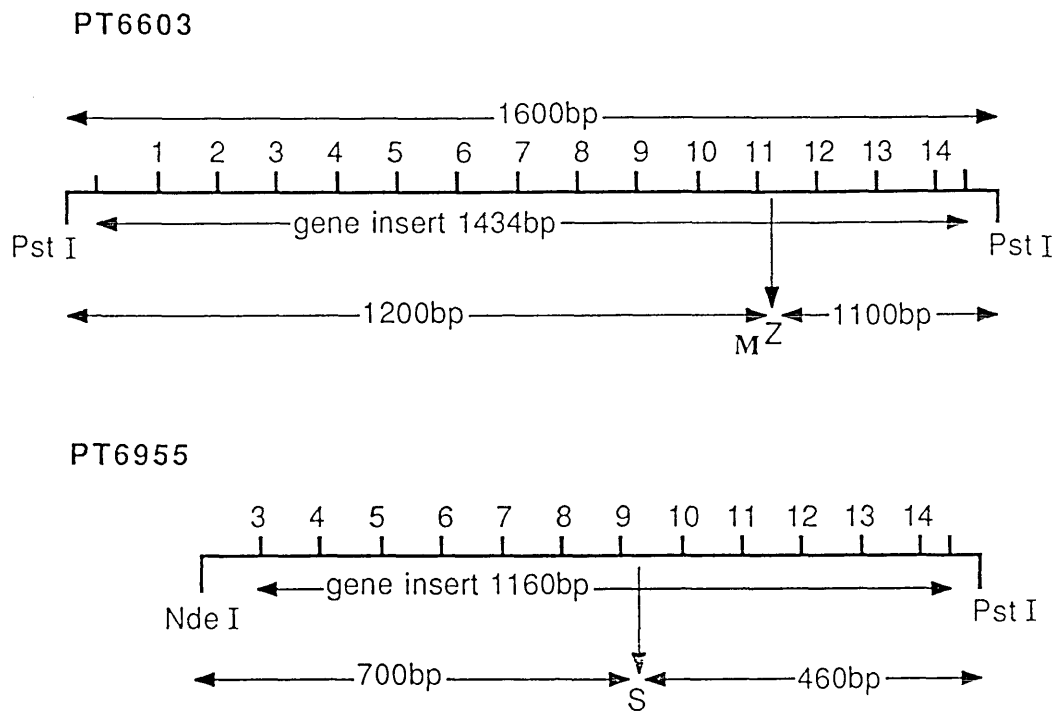


FIGURE 22 CLONED HUMAN DNA SEQUENCE INSERT FOR THE β GLOBIN GENE

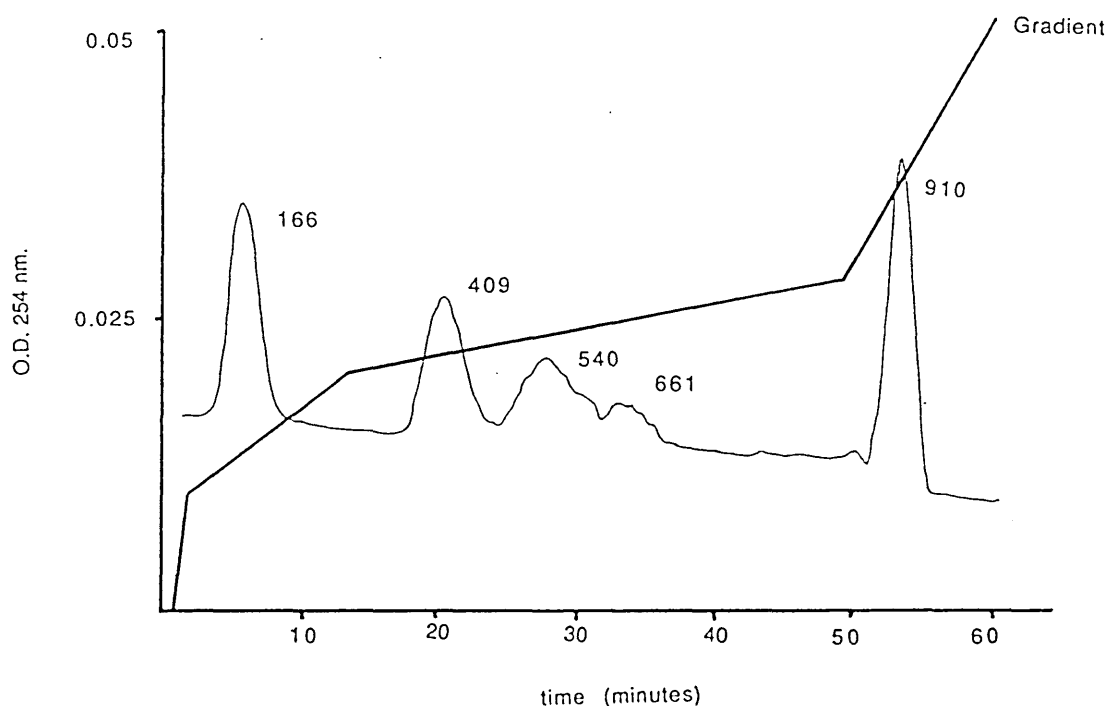
The restriction map of the normal β -globin gene sequence insert found in H β f5. It is flanked by two *Bam HI* restriction sites. The insert codes for the 5' end of the human β -globin gene. The sickle mutated β -globin gene insert of H β 1-S differs from the normal sequence by a single base change at the sickle site. The insert is also considerably longer than that of H β f5 (an additional 2300bp) and is flanked by two *Pst I* restriction sites .



Cloned DNA sequence inserts for the Alpha-1-Antitrypsin gene

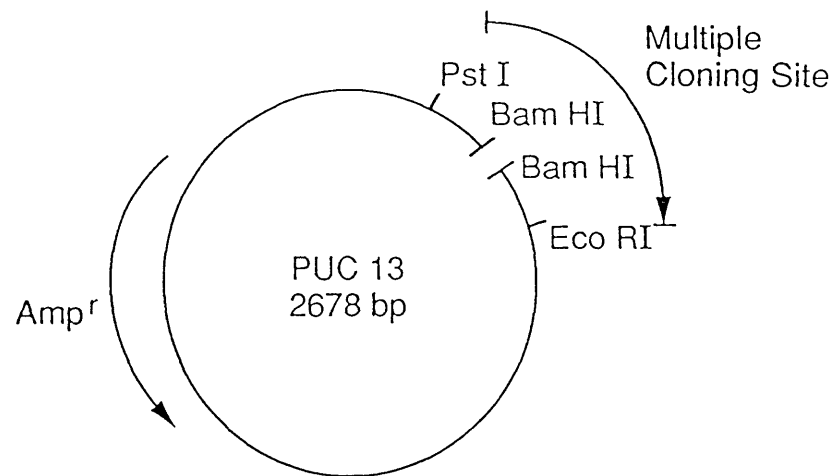
FIGURE 23

A. is a restriction map of the cDNA sequence of the alpha-1-antitrypsin gene, which is found as the DNA insert of pTG603. The cDNA was isolated from a human liver cDNA bank and was constructed in PBR322 by G-C tailing into the *Pst I* site (Courtney, 1985). It contains the complete alpha-1-antitrypsin cDNA sequence (apart from a few bases at the 5' end) and has the normal M sequence at the Z and S loci. B. is a restriction map of the insert found in pTG955, which codes for a part of the cDNA sequence of alpha-1-antitrypsin. The insert was generated by site directed mutagenesis of pTG603 at the S locus. The restriction sites used for cloning this insert into a PBR322 vector were *Pst I* and *Nde I* (Courtney, 1985).

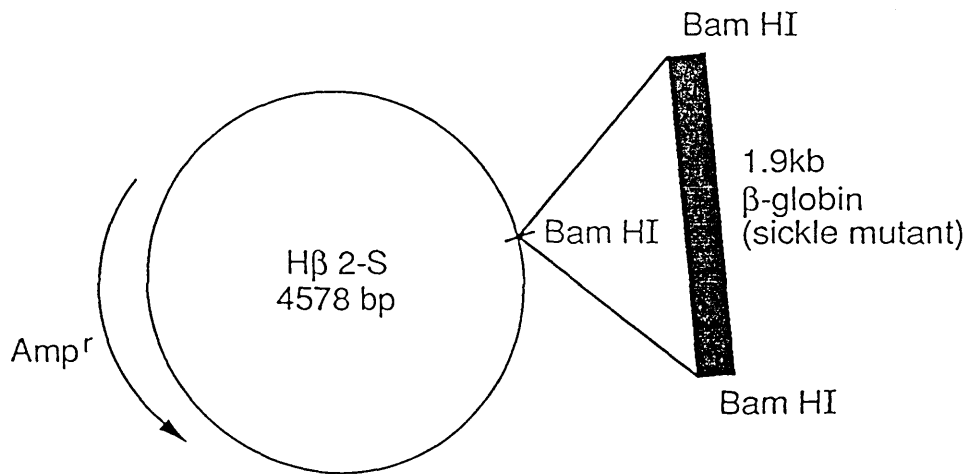


THE PURIFICATION OF RECOMBINANT DNA RESTRICTION FRAGMENTS USING FPLC AND AN ION-EXCHANGE COLUMN.

FIGURE 24 Chromatogram showing the resolution of five restriction fragments (910 bp, 661 bp, 540 bp, 409 bp, 166 bp) from a *Dde I* digest of a recombinant plasmid, on an ion-exchange column (Mono Q HR 5/5, Pharmacia Ltd.). The salt gradient used was between 0.8 M NaCl and 0.85 M NaCl, run at a flow rate of 0.5 ml/min over a period of one hour. The maximum loading capacity for this separation was 50 μ g (2 O.D.254).



A. Bam HI Digested PUC 13 with Dephosphorylated ends



B. Recombinant Plasmid H β 2-S

FIGURE 25 Restriction Enzyme Maps

similar in size and theoretically bore little sequence homology with the detector probes to be used.

To facilitate cloning of the sickle gene, the insert was isolated from the pBR322 vector of Hb1-S and ligated into a pUC vector. To do this the gene insert was excised from the Bam HI restriction site, purified from the vector by electroelution, and ligated into the Bam HI site of a pUC 13 vector. This high copy number vector, Hb2-S (depicted in figure 25) facilitated increased production of the sickle gene through cloning.

3.2 Differential hybridisation between pairs of oligonucleotides with single base changes

The pairs of oligonucleotides to be used as detector probes in this study were initially tested for their differential melting behaviour. The empirically derived formula by Wallace et al. (1979), used for estimating the dissociation temperature (T_d) of oligonucleotide sequences was applied to set the approximate range of hybridisation temperatures to be tested for each oligonucleotide pair. Parameters affecting the melting temperature of an oligonucleotide were otherwise kept constant, for example ionic strength and other destabilising factors.

The optimum hybridisation temperature, which ultimately resulted in the greatest difference in duplex dissociation between matched and mismatched oligonucleotides, was determined for each pair of detector probes. This was carried out through a series of dot-blot filter hybridisations, followed by stringent washes at increasing temperatures and constant salt concentrations. The final determination of melting temperatures was estimated from the exposure of the radioactively Kinase end-labelled probes to X-ray sensitive film. To

confirm the results obtained, an accurate determination of the melting temperature (T_d) was also made for some randomly selected pairs of oligonucleotides by scanning the density of these exposures using a densitometer.

Melting temperatures for both the beta-globin and alpha-1-antitrypsin coding oligonucleotide detector probes were determined in this way. The sequences of these probes are listed in table 7.

A third novel gene probe directed towards the beta-globin gene was also tested for its melting behaviour. This probe was designed and synthesised by Chollet and was expected to have altered hybridisation properties. This probe contained a 2-aminoadenine 1(2,6-diaminopurine) residue in place of the normal adenine moiety at the mutation site of the normal beta-globin gene. This analogue had previously been shown to increase the stability between a thymine: 2-aminoadenine base pair. An adenine:2-aminoadenine base pair on the other hand displayed less hybrid stability. (Chollet et al 1988)

3.2.1 Alpha-1-antitrypsin Z and S mutations

The melting temperatures for the Z and S mutant specific oligonucleotide pairs were established using linearised plasmid DNA: pTG 603 (genotypically normal at both the S and Z loci) and pTG 955 (containing the mutated S locus) respectively. The dot-blots were carried out on nitrocellulose, immobilising the denatured single-stranded plasmid DNA containing the alpha-1-antitrypsin gene sequences. The single base mismatches between the normal oligonucleotides and the mutated complementary genes, designed to give the greatest hybrid instability, were a C:A mismatch in the Z allele and a T:T mismatch in the S allele. The mismatches between the mutant

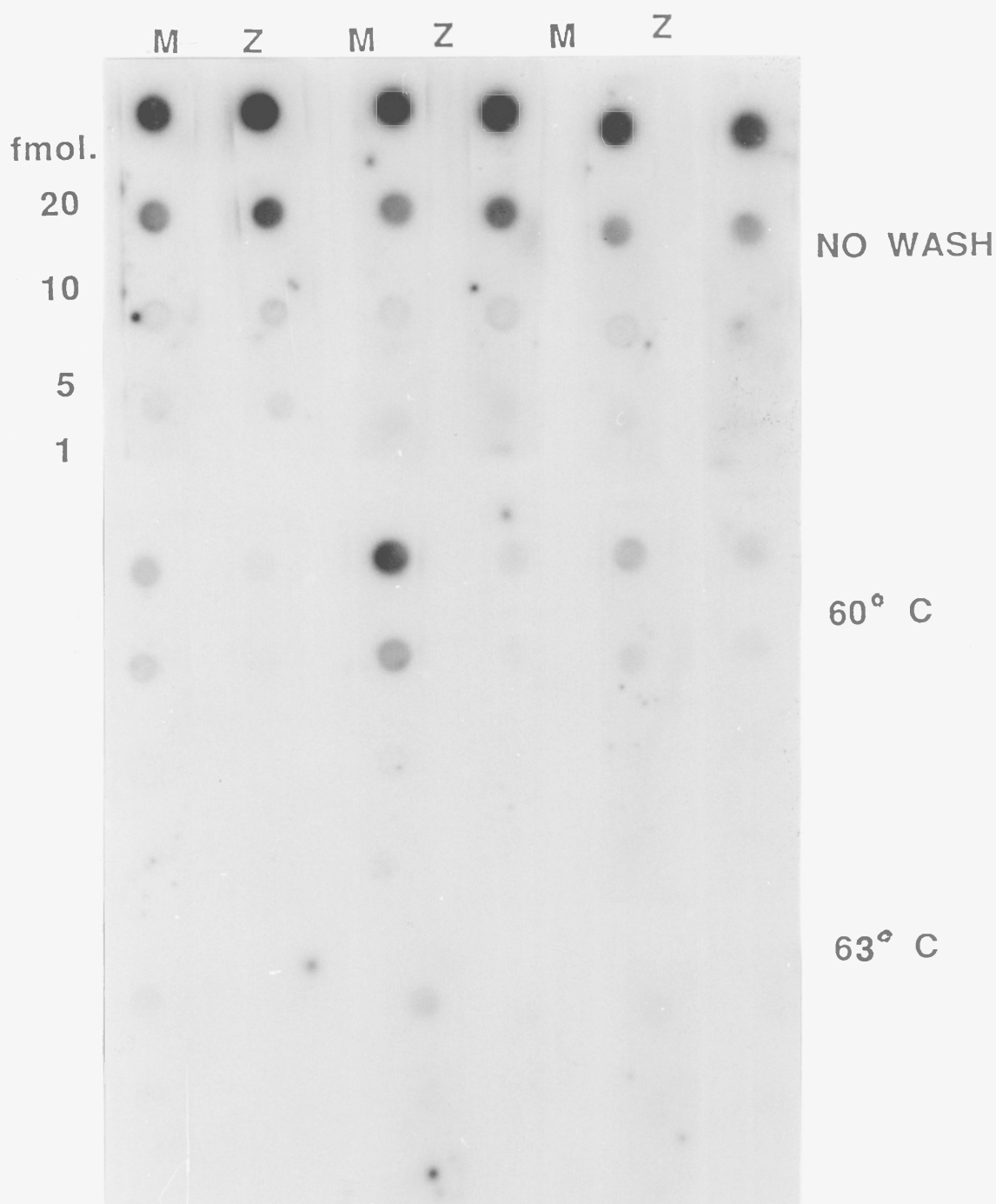


FIGURE 26 DIFFERENTIAL HYBRIDISATION OF M AND Z OLIGONUCLEOTIDE PROBES TO THE LINEARISED α_1 -ANTITRYPSIN GENE INSERT IN pTG 603.

Three hybridisation temperatures 45° C, 50° C, 55° C were compared along with different post hybridisation washing temperatures: 58° C, 60° C, 63° C. Using 20mer M and Z (10 pmol ^{32} P kinase end-labelled) oligonucleotide probes and linearised target DNA (20fmol, 10fmol, 5fmol, 1fmol) immobilised to nitrocellulose, overnight hybridisations were set up. The results show that the best differentiation between the hybridisation of matched and mismatched oligonucleotide was achieved at a hybridisation temperature of 50° C. and a 10 minute post hybridisation wash at 60° C.

oligonucleotides and the normal complementary gene on the other hand were G:T and A:A respectively. The estimated and the experimentally determined melting temperatures are shown in table 9.

Using the protocol described in section 2.3.9.3 of the methods, hybridisation at 50°C for the Z specific (figure 26) and 53°C for the S specific oligonucleotide pairs was found to result in the greatest differentiation between the melting temperatures. Under these conditions it was found that the dissociation of the mismatched hybrid was lower than the estimated melting temperature. The overall difference in melting temperatures between each pair of oligonucleotides was 3°C for the Z and 2°C for the S specific sequences examined.

3.2.2 Beta globin sickle mutation

The normal and sickle gene clones, Hbf 5 and Hbl-S were used to determine the melting temperatures of the complementary oligonucleotide pair. The same procedure was used as that for the measuring of melting temperatures of the alpha-1-antitrypsin gene. The mismatch generated between normal and mutant sequences was either A:A or T:T. The recorded melting temperatures between mismatched sequences were found to be 4-6°C lower than expected (see table 9). This difference in melting temperature, also observed in experiments with the alpha-1-antitrypsin gene suggested that it was not only the loss of the thermal stability of a hybrid complex that affected melting temperatures. Certain base mismatches affected the degree of hybrid stability more than others. In this way the results were in agreement with data from NMR studies by Arnold et al (1987), where it was shown that A:A mismatches were more unstable than T:T mismatches. Mismatches between G:N nucleotides (where N equals any of the four bases)

however, were generally more stable than other mismatches.

The optimum hybridisation temperature for the maximum differential melting behaviour was found to be 50°C. A difference of approximately 2°C could be established between the melting behaviour of the oligonucleotide sequence pair hybridised to the normal beta-globin target DNA (T:T mismatch). A differential melting temperature of 4°C was recorded between the hybridisations of the oligonucleotides to the sickle mutated target DNA. (A:A mismatch)

3.2.3 2-aminoadenine analogue

A comparison between the melting behaviour of the normal beta-globin oligonucleotide with the 2-aminoadenine analogue containing oligonucleotide was made. In place of the normal adenine residue at the sickle cell mutation site this oligonucleotide contained the 2,6-diaminopurine, D analogue, known to stabilise the hybrid duplex under matched conditions with its additional hydrogen bond. Under mismatched conditions however the additional bonding site induces increased destabilisation of the hybrid duplex (the structure is shown in figure 11).

The hybridisation temperature was maintained at 50°C for both hybridisations of the novel detection probe to the normal and mutated beta-globin gene sequences. The presence of a third hydrogen bonding site of the 2-aminoadenine containing oligonucleotide resulted in an increased melting temperature of 66°C (figure 27). The mismatched hybrid complex however displayed a melting temperature of 56°C, which was unchanged from that recorded for the normal 20mer oligonucleotide. This presented a total difference of 10°C between the dissociation temperatures of the matched and mismatched sequences. The presence of a third hydrogen bond therefore increased the hybrid

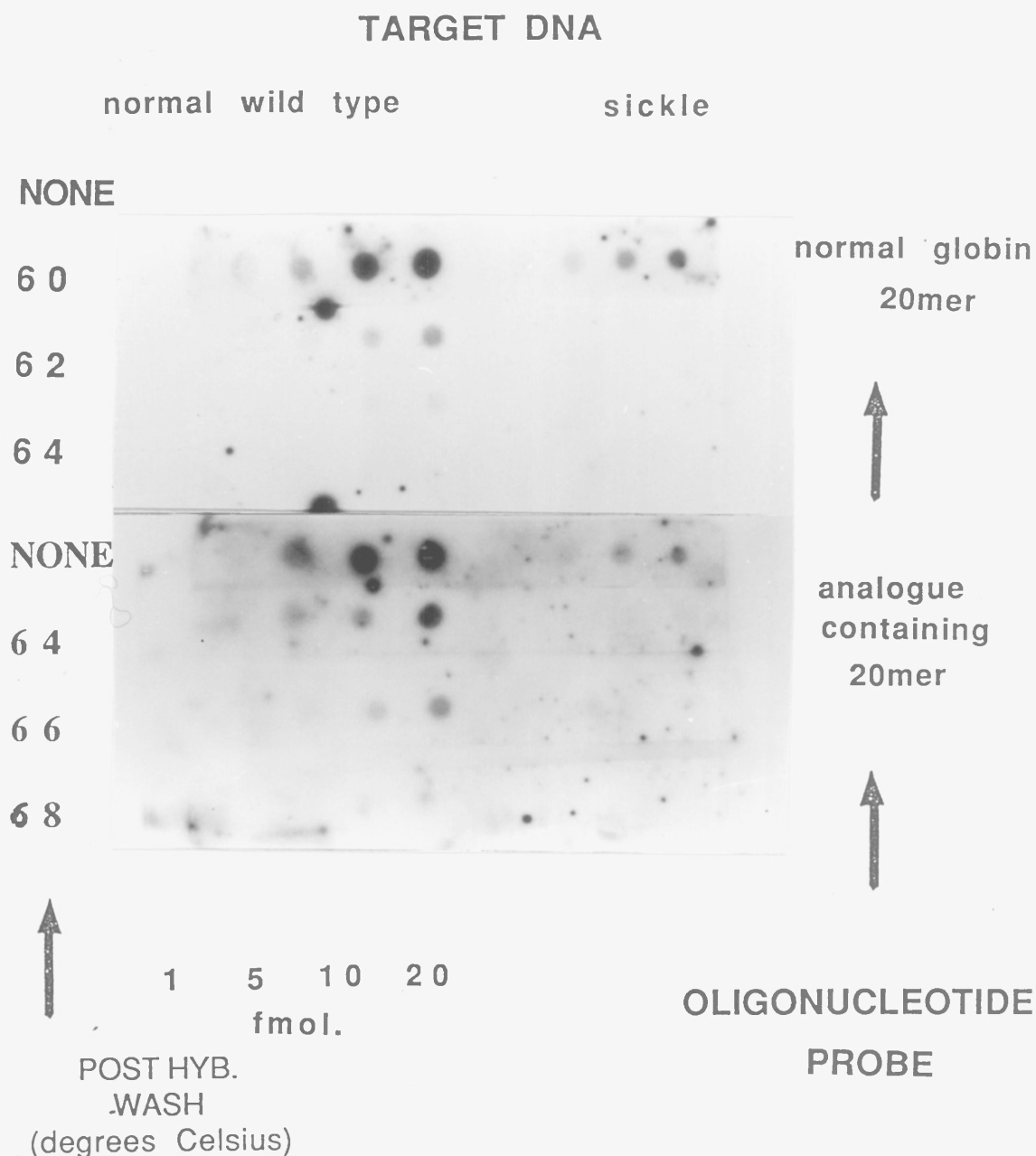


FIGURE 27 A COMPARISON BETWEEN THE MELTING BEHAVIOUR OF NORMAL B-GLOBIN OLIGONUCLEOTIDE AND THE ANALOGUE CONTAINING OLIGONUCLEOTIDE

Hybridisations were performed overnight at 50 °C using 20 fmol, 10 fmol, 5 fmol and 1 fmol immobilised and linearised H β f5 and H β 1-S target DNA. Post hybridisation washes were carried out for 10 minutes at a range of temperatures. The results demonstrate a 6° C increase in melting temperature of the 2-aminoadenine containing oligonucleotide probe when compared to the melting behaviour of the normal 20mer probe from a matched hybrid duplex.

Oligonucleotide melting temperatures

<u>Oligonucleotide</u>	<u>mismatch</u>	<u>Estimated Td</u>	<u>real Td</u>	<u>difference in Td</u>
<u>Hybridisation to normal target</u>				
Z normal		62	62	
Z mutant	G:T	60	59	3
<u>Hybridisation to mutated target</u>				
S normal		58	56	
s mutant	T:T	58	58	2
<u>Hybridisation to normal target</u>				
Globin normal		62	60	
sickle	T:T	62	58	2
<u>Hybridisation to sickle target</u>				
Globin normal	A:A	62	56	
Sickle		62	60	4
<u>Hybridisation of analogue</u>				
Globin target		63	66	
sickle target	A:A	63	56	10

TABLE 9 The pooled oligonucleotide melting temperature results, established from dot-blot hybridisations for pairs of 20mer oligonucleotide detector probes coding for the Sickle, Z and S loci specific mutation sites, as well as the normal sequences associated with the β -globin and the α_1 -antitrypsin genes. The melting temperature was taken to be the temperature at which half of the probe dissociated from the target DNA under stringent washing conditions.

stability by a melting temperature of 6°C.

3.2.4 Summary

1. For both the beta-globin and alpha-1-antitrypsin specific oligonucleotide detector probes the optimum temperature for the maximum differential hybridisation to target DNA was 5-12°C lower than the estimated melting temperature of each probe.
2. The melting temperatures of the oligonucleotides were influenced by the base composition at the site of mismatch within a hybrid duplex. A mismatch between two thymines (T:T) showed more signs of hybrid stability than a mismatch between two adenines (A:A).
3. The presence of a third hydrogen bond between a 2-aminoadenine and a thymine residue of opposite DNA strands raised the melting temperature of a 20mer oligonucleotide by 6°C. The presence of a third hydrogen bonding site however, did not affect the melting temperature between an A:A mismatch.

3.3 The detection of single copy genes

To determine whether oligonucleotide detector probes would be able to detect single copy genes in a sandwich hybridisation system, conventional hybridisation experiments were set up. In these experiments the target nucleic acid was immobilised to a solid support and detection occurred using 32 P Kinase end-labelled 20mer oligonucleotide probe contained in the hybridisation solution. The optimum oligonucleotide labelling system was established for the

detection of single copy genes in human DNA. The gene used was alpha-1-antitrypsin and the detector probe pair were the 20mers synthesised to be specific for this gene around the Z locus. It was thought that the limits of detection encountered through these hybridisation experiments would be similar to those one could expect in a sandwich assay.

3.3.1 A comparison of Membrane and direct Agarose gel hybridisations.

Experiments were designed to test for the detection limits of end-labelled oligonucleotide probes when hybridised to immobilised, single stranded and linearised plasmid DNA.

Two hybridisation strategies were approached for this analysis: Southern blotting and direct agarose gel hybridisation. Direct gel hybridisations were found to be five to ten times more sensitive in detecting DNA sequences than Southern blotting on nitrocellulose. This became evident when it was found that a considerable amount of DNA was lost during Southern blotting. DNA transfer from the agarose gel to nitrocellulose appeared to be more than 80% efficient. However, as less than 80% of the DNA was present after transfer, the loss in sensitivity of Southern blotting must have been due to a loss of target DNA, from the filter during hybridisation. This is illustrated in figure 28. These results were later confirmed by the findings of Thein et al (1986) where a five fold loss in sensitivity was also recorded. Gingeras et al (1987) also quantified the loss of target DNA from nitrocellulose filters as being approximately 80%.

Oligonucleotide probes were Kinase end-labelled with nucleotides containing ^{32}P with specific activities of 3000 $\mu\text{Ci}/\text{mMol}$ and hybridised to gel immobilised target DNAs. These were generally found to display limits of detection of around 50 attomoles (figure

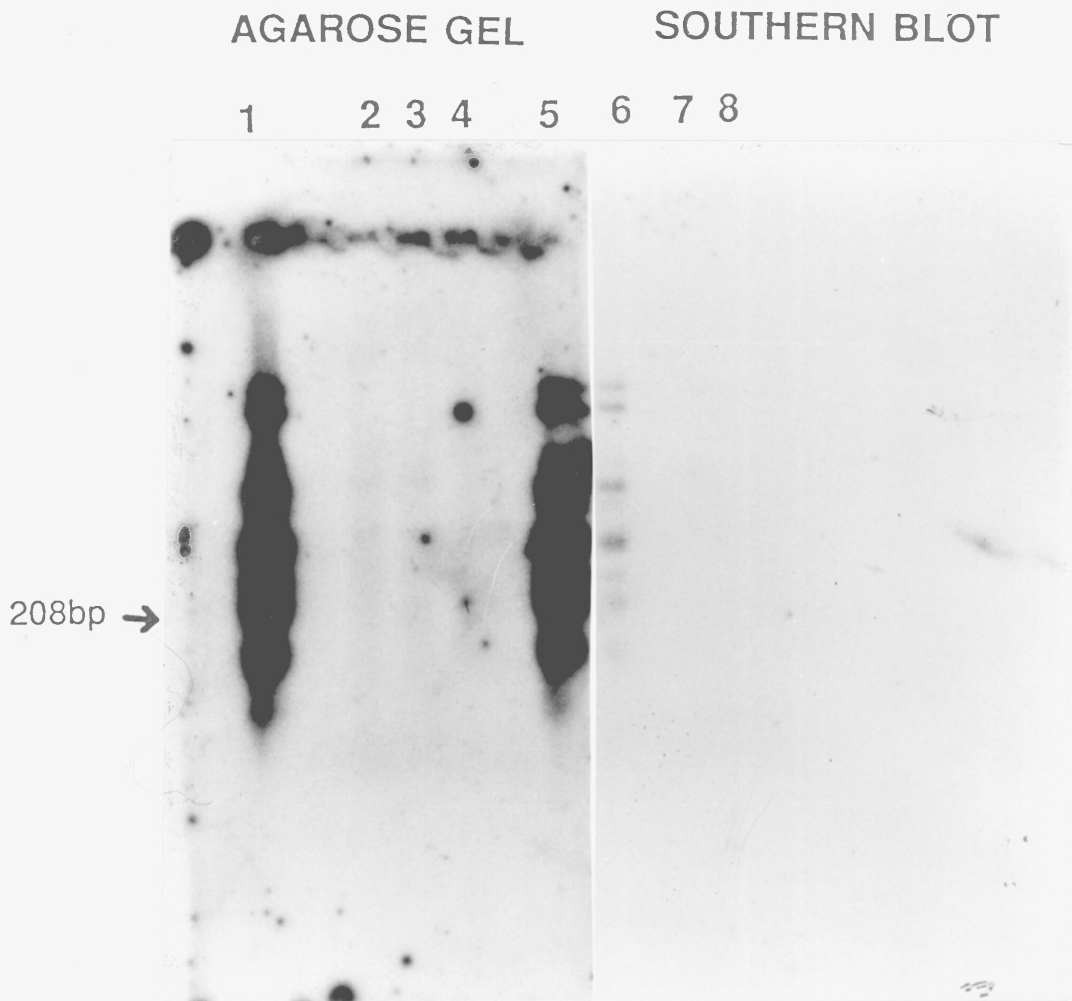


FIGURE 28 A COMPARISON BETWEEN THE DETECTION LIMITS OF HYBRIDISATIONS PERFORMED ON DRIED AGAROSE GELS AND NITROCELLULOSE FILTERS

Lanes 1, 4, and 5 contain ^{32}P kinase end-labelled ϕX174 *Hae III* restriction digested DNA. Lanes 2, 6, and 3, 7, contain 15 μg and 25 μg respectively of *Hae III* restriction digested Human DNA. Hybridisations were carried out using 6 pmol β -globin 20mer oligonucleotide ^{32}P kinase end-labelled to a specific activity of approximately 1.1×10^9 dpm/ μg . The results demonstrate the loss of target DNA during the transfer from agarose to nitrocellulose during Southern blotting. This can be seen by the reduced transfer of ϕX174 marker DNA. The results also demonstrate a difference in detection limits. In A, the 208bp of the β -globin gene fragment expected to be complementary to the 20mer can almost be detected whereas in B, no human DNA is visible.

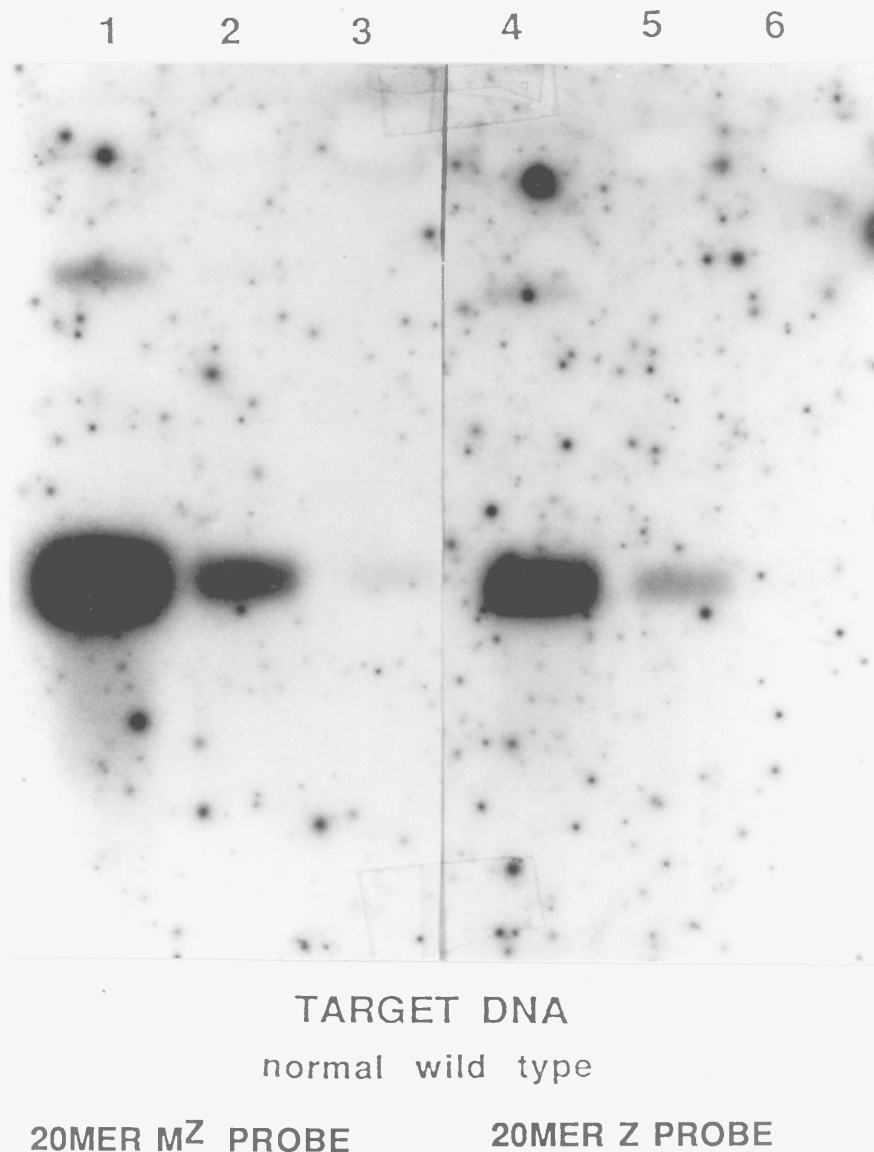


FIGURE 29 DIRECT AGAROSE GEL HYBRIDISATION OF 20MER OLIGONUCLEOTIDES CODING FOR THE M^Z AND Z GENOTYPES TO RECOMBINANT TARGET DNA CONTAINING THE α_1 -ANTITRYPSIN GENE SEQUENCE (PTG 603)

The 1.6kbp α_1 -antitrypsin gene insert of PTG 603 was isolated through *Pst* I restriction digestion. 20mer Oligonucleotides coding for both the normal (M^Z) and the Z gene sequence were ³²P kinase-end-labelled to a specific activity of approximately 8.3×10^8 dpm/ug. and were hybridised to the (dried) gel immobilised target DNA. Lanes 1., 4., and 2., 5., and 3., 6., contain 3.5 fmol, 0.35 fmol, and 0.035 fmol. of target DNA respectively. Upon hybridisation, overnight at 50^o C the gels were washed stringently at 60^o C and autoradiographed overnight on X-OMAT X-AR 5 film. The results reveal a detection limit of 0.035 fmol for matched duplexes.

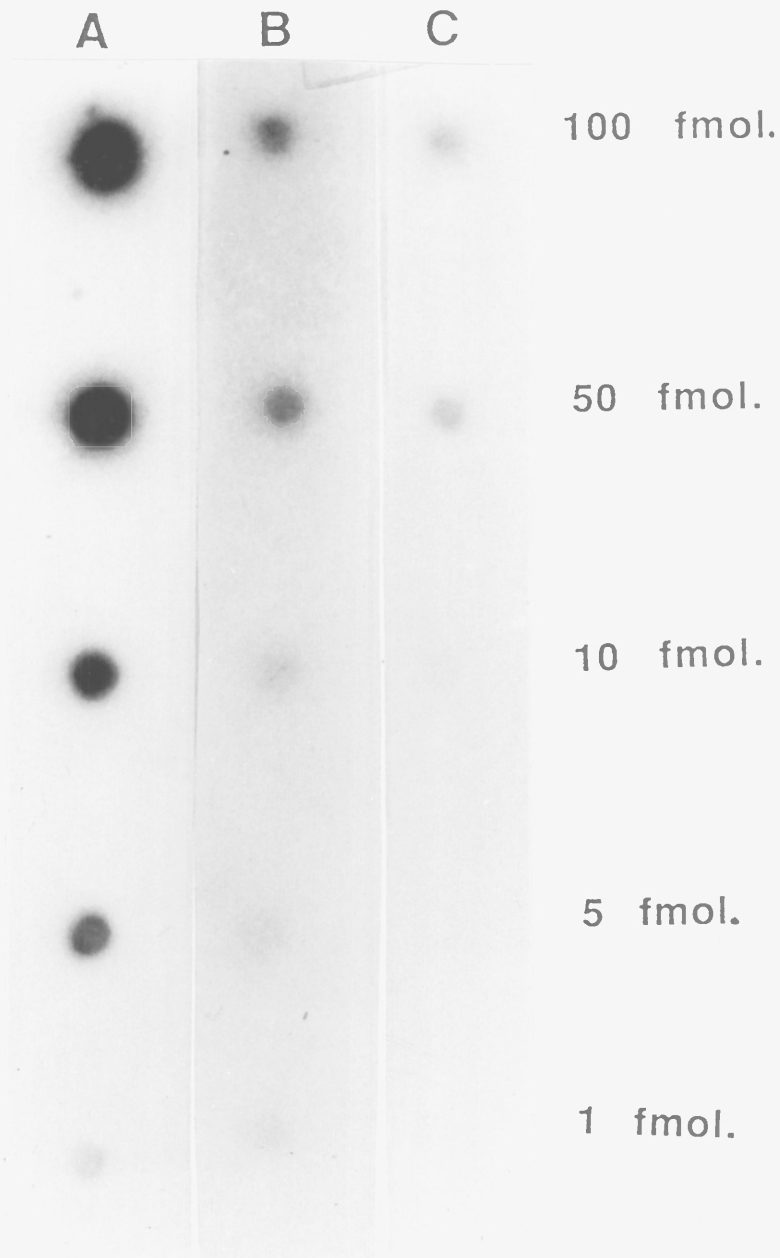


FIGURE 30 OLIGONUCLEOTIDE RETENTION PROPERTIES OF ZETA PROBE FILTER PAPER

Oligonucleotides kinase end-labelled with ^{32}P were applied to Zeta probe filters in concentrations of 100 fmol., 50 fmol., 10 fmol., 5 fmol. and 1 fmol. and immobilised through baking at 80°C for 2 hours. The filters were then exposed to conditions simulating those of an overnight hybridisation reaction at 50°C . A. represents the filter unexposed to any treatment. B. was exposed to hybridisation and a single non-stringent wash. C. has been exposed to hybridisation and a stringent 10 minute wash at 63°C . The results demonstrate a large loss of immobilised oligonucleotide particularly after hybridisation. They also suggest that there is a maximum loading capacity of around 300 pg./dot.

29). The limits of detection after Southern blotting were approximately ten times greater. In order to detect single copy genes such as alpha-1-antitrypsin or beta-globin, a ten fold increase in sensitivity was required over the limits found through direct gel hybridisations. Such a sensitivity would detect a minimum of 5 attomoles which would be equivalent to the concentration of single copy genes present in 10 ug of human genomic DNA.

The nylon membrane Zeta probe was used to compare the retention properties of immobilised oligonucleotides with those of longer DNA fragments on nitrocellulose. The properties of this nylon membrane were reported to be suited for the immobilisation of oligonucleotides, by the manufacturers. Through mock dot-blot hybridisations with ^{32} P Kinase end-labelled and filter immobilised oligonucleotide probes a considerable loss of DNA was found to occur. The results are illustrated in figure 30. This finding was in support of the observation that filter-based hybridisation systems were not suited to single copy gene detection.

3.3.2 Sensitivity

The problem of increasing the limits of detection with oligonucleotides was approached in four ways. Initially the sensitivity could be increased by isolating the radioactively labelled oligonucleotide products from the Kinase reactions and using only these probes in hybridisation reactions. This was achieved by separating the labelled from the unlabelled oligonucleotides through acrylamide gel electrophoresis. The ^{32} P labelled oligonucleotides migrated more slowly through the gel matrix as a result of the extra phosphate group and could be excised and eluted from the gel. When incorporated into a hybridisation reaction this purification resulted

in a greater proportion of labelled hybrid complexes and increased the limits of detection by about two fold.

A second method of increasing the detection limits was to directly increase the specific activity of the labelled oligonucleotide probe. Nucleotides containing ^{32}P isotopes with a specific activity of 7000 $\mu\text{Ci}/\text{mmol}$ were used to Kinase end-label the oligonucleotides. This together with the separation of the unlabelled products from the labelled resulted in a 5 fold increase in the detection of target DNA. This sensitivity was in fact sufficient to detect the alpha-1-antitrypsin gene in a total of 15 μg of human genomic DNA.

A third approach was investigated which would also increase the sensitivity, this involved oligolabelling the oligonucleotide probe, using the large Klenow fragment of DNA polymerase I. By incorporating several radioactively labelled nucleotides into the oligonucleotide sequence even the substitution of a single base would increase the specific activity of the probe. Using a 6mer primer for this reaction complementary to the 3' end of the 20mer oligonucleotide probe, it was found that the yields of new strand synthesis were low (figure 31). Furthermore, unless several different bases were substituted with radioactively labelled nucleotides the overall specific activity was no more than that obtained by ^{32}P end-labelling with the higher specific activity (^{32}P dATP, 7000 $\mu\text{Ci}/\text{mmol}$) radioisotope. This was due to the alpha-1-antitrypsin 20mer oligonucleotide, serving as the template to synthesis containing only two thymine sites, which could be replaced with the radioactively labelled adenine containing nucleotide on the new strand.

Finally a method of increasing specific activity by end-tailing the oligonucleotide with several radioactively labelled nucleotides was approached. Work by Woodhead et al showed that the 5' end tailing of

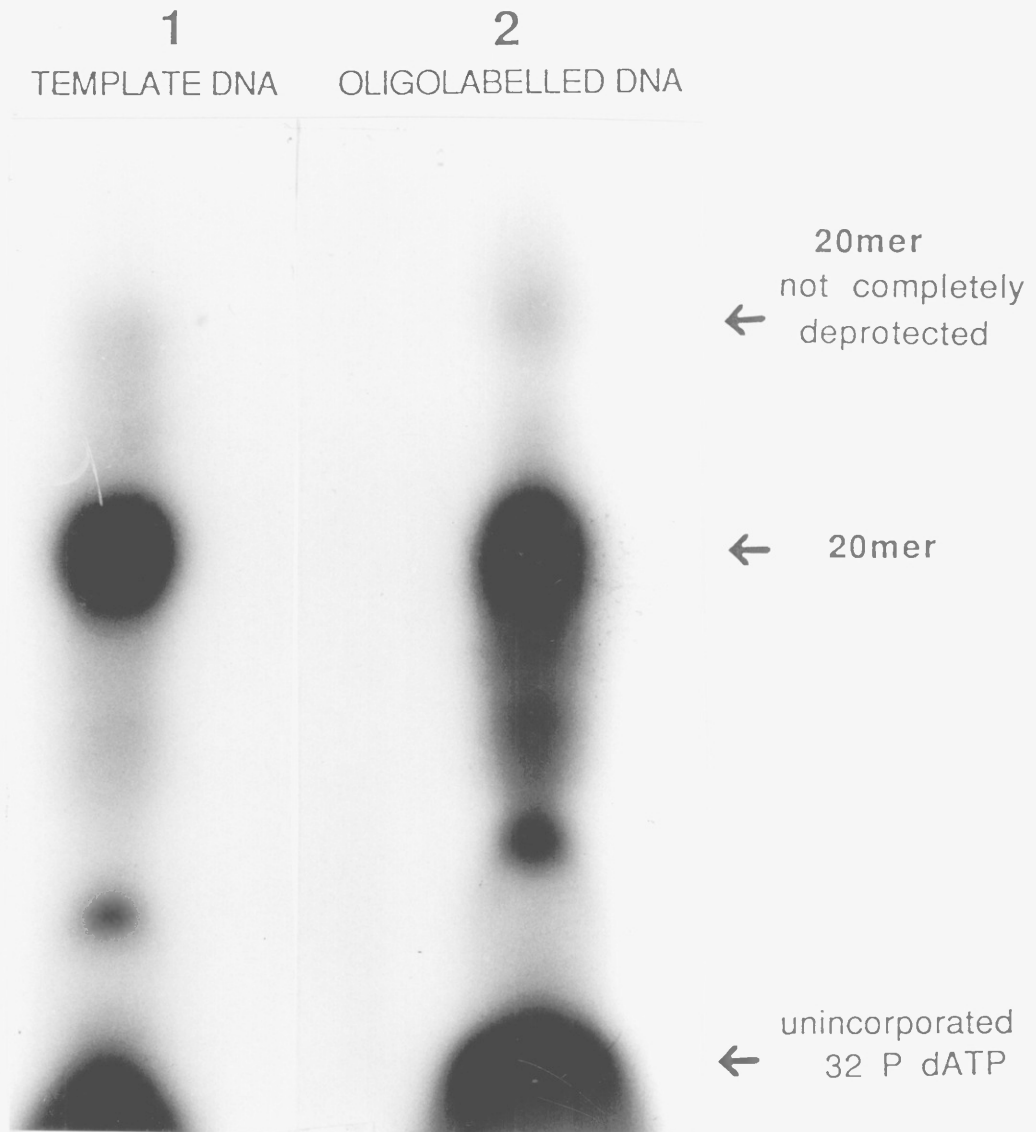


FIGURE 31 POLYACRYLAMIDE GEL ELECTROPHORESIS FOR THE CHARACTERISATION OF AN OLIGOLABELLED 20MER OLIGONUCLEOTIDE

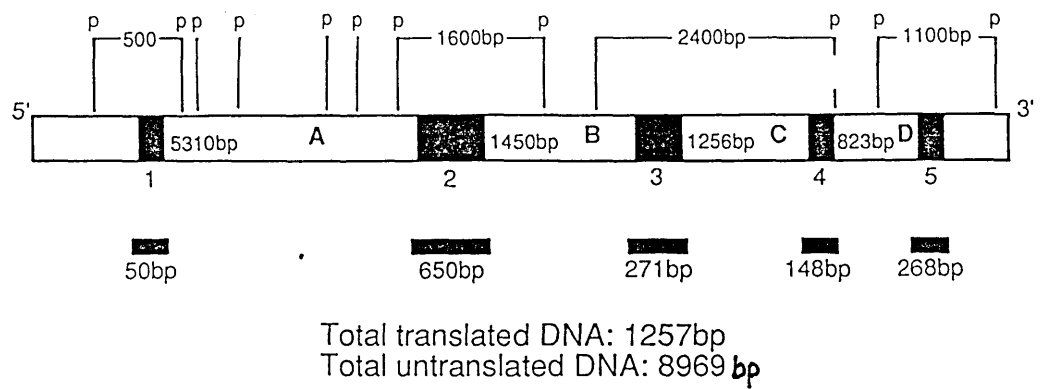
Lane 1. contains ^{32}P kinase end-labelled 20mer "template" oligonucleotide (20ng, 3.3 pmol) coding for the Z locus of the $\alpha 1$ -antitrypsin gene (specific activity, 4×10^8 dpm/ug). Lane 2. contains the oligolabelling reaction products, including 20ng of the labelled oligonucleotide template DNA. A comparison of the specific activities present in the gel, through Cerenkov counting revealed an increased activity in lane 2. (1.06×10^9 dpm/ug). From the results however, it can be seen that most of these counts are derived from incomplete reaction products, having failed to elongate to the length of a 20mer.

20mer oligonucleotides had little effect on the hybridisation properties to target DNA, if the tail length was kept to 15 bases. In this work a 15mer tail was added to the oligonucleotide during synthesis. Difficulties were however encountered with this approach when terminal transferase was used as a 3' tailing enzyme. Keeping the tail length down in size was difficult to control, even when the nucleotides in solution were limiting to the reaction. The repeatability of such a reaction was also limited by the activity of the enzyme. Such a labelling technique might only be feasible if the radioactive nucleotides could be incorporated during synthesis of the oligonucleotide.

3.3.3 Detection of the alpha-1-antitrypsin gene in the human genome

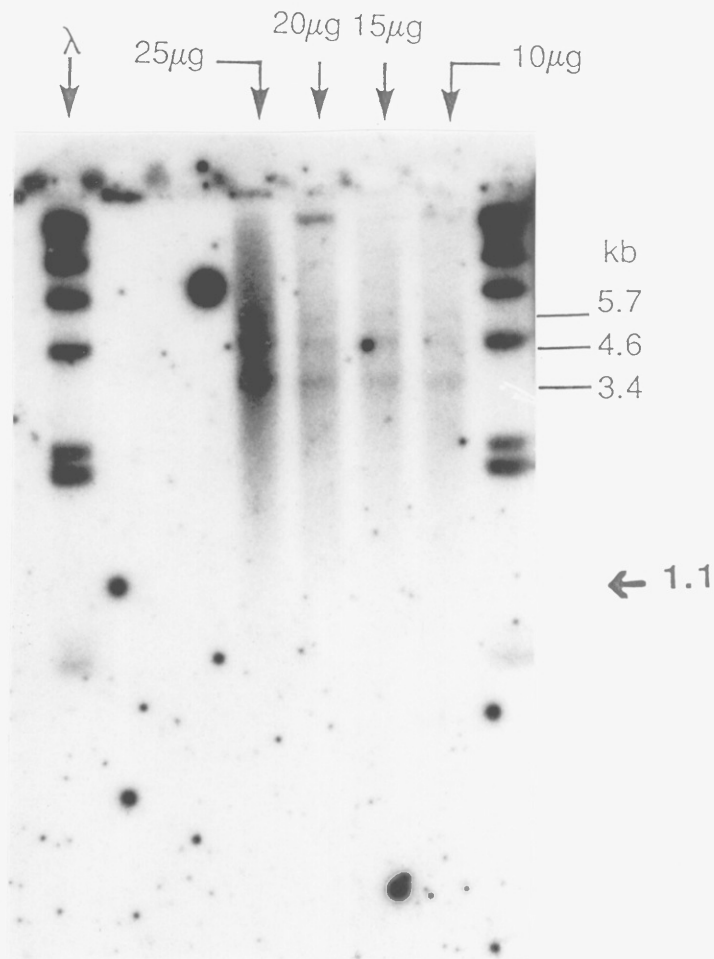
The single copy gene of alpha-1-antitrypsin was detected in human samples of genotypically normal DNA, using ^{32} P Kinase end-labelled oligonucleotides of high specific activity and using oligolabelled DNA probes. The genomic DNA was digested with a Pst I restriction enzyme which generated 4 restriction fragments within the alpha-1-antitrypsin gene, of the following sizes: 2.4, 1.6, 1.1, 0.5 kbp (illustrated in figure 32). Exon V containing the sequence of DNA susceptible to mutation at the Z locus, was present in the 1.1 kbp restriction fragment. Following electrophoresis through a 1% agarose gel the enzyme restricted DNA was used for direct gel hybridisations.

An oligolabelled probe was generated from the pTG603 recombinant plasmid using the large Klenow fragment of DNA polymerase I. Direct gel hybridisations with this probe revealed all but the smallest fragment of the alpha-1-antitrypsin gene. The probe was oligolabelled to a specific activity of 2×10^9 dpm/ug and could detect fragments in 10 ug DNA (5 attomoles of single copy genes).



Genomic DNA Sequence for Human Alpha-1-Antitrypsin

FIGURE 32 A restriction map of all the *Pst* I sites within the α_1 -antitrypsin gene. The Z allele is contained within a 1100bp *Pst* I fragment and the S allele in a 2400bp restriction fragment. The sites for this map have been derived from data by Long et al. (1984).



Detection of the normal alpha₁ antitrypsin gene using a 20 mer oligonucleotide probe

FIGURE 33A ³²P kinase end-labelled oligonucleotide detector probe was used in this direct gel hybridisation to *Pst* I digested human DNA, isolated from peripheral blood cells. The oligonucleotide was the 20mer (15 pmol) complementary to the Z locus of the α₁-antitrypsin gene, labelled to a specific activity of approximately 2.8×10^9 dpm/ug. The oligonucleotide was expected to detect the 1100bp fragment containing the Z allele under stringent hybridisation conditions. These conditions included an overnight hybridisation at 50° C, followed by several post-hybridisation washes at 50° C. The results show three bands ranging in size from 5.7 kb., to 3.4 kb. Washing temperatures could be raised to 64° C before these bands were removed, suggesting that these were specific hybridisations. As the fragment sizes are all greater than the expected size of 1.1kb., they were probably generated by incomplete restriction enzyme digestion. The melting temperature suggested that the DNA sample tested (at various dilutions) was from a genotypically normal (M^Z) individual.

The 1.1 kbp fragment could not be detected easily when the same hybridisation reaction was carried out with ^{32} P Kinase end-labelled Mz 20mer oligonucleotide. This was primarily due to the difficulties in producing end-labelled probes with sufficient specific activity.

In one experiment, however, three bands were clearly visible with the following fragment lengths: 5.7, 4.6 and 3.4 kbp. (the specific activity of the probe was 2.8×10^9 dpm/ug). The result is depicted in figure 33. The bands detected were probably generated by an incomplete restriction enzyme digest of the genomic DNA. Increasing the stringency of washes by raising the buffer temperature did not remove these bands until 64°C was reached. This suggested that a specific hybridisation between oligonucleotide and genomic sequence had occurred.

From these hybridisations experiments it became apparent that single gene detection using oligonucleotide probes, was not going to present an easy method for diagnosis. In a sandwich hybridisation assay similar complications would be encountered making diagnosis difficult. The problem of sensitivity was overcome with the development of the polymerase chain reaction by Saiki et al (1986) in later work. This reaction could specifically amplify a sequence of interest to concentrations easily detectable using end-labelled oligonucleotides. The hybridisation results for single gene detection using this method are described in section 3.7.

3.3.4 Summary

1. When comparing membrane with agarose gel hybridisations the latter proved to be ten times more sensitive in detecting specific DNA sequences.
2. The specific activity of the radiolabelled oligonucleotide was the

limiting factor with respect to single gene detection.

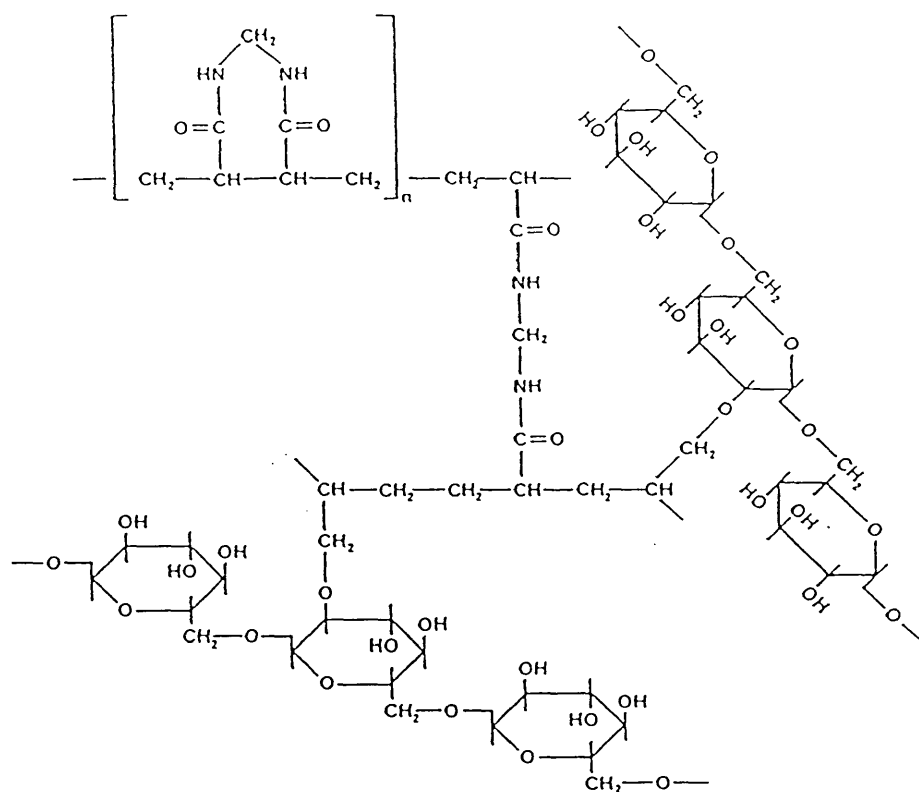
3. Increasing the specific activity of an oligonucleotide probe could be carried out using a number of techniques, none of which were entirely satisfactory. Single genes could however be detected using these probes.
4. Single gene detection in the human genome using oligonucleotides in a direct hybridisation was not easily carried out. For diagnostic purposes an amplification of either target DNA or a dramatic increase in specific activity of the probe was needed.

3.4. Immobilisation studies

Oligonucleotides were immobilised by three different methods to solid supports;

- a) by carbodiimide activation (Allfrey and Inoue, 1978),
- b) enzymatically (Goldkorn, 1986)
- c) by diazotization (Bunemann, 1982, a)).

These methods, originally developed for the attachment of DNA restriction fragments to solid support matrices, were adapted and tested for their suitability in immobilising oligonucleotides. The first two methods relied on 5' covalent end linkage and the last method was achieved by non-covalent random base linkage. Calf thymus DNA sonicated to an average size of 300bp and beta-globin oligonucleotides ranging in size from 20bp to 100bp were used in these studies. The oligonucleotide sequences are listed in table 7. For each method a range of solid supports was tested, and the optimum support



HYPOTHETICAL PARTIAL STRUCTURE OF SEPHACRYL

(Reproduced from the Pharmacia Gel filtration booklet with permission)

Sephacryl S-1000

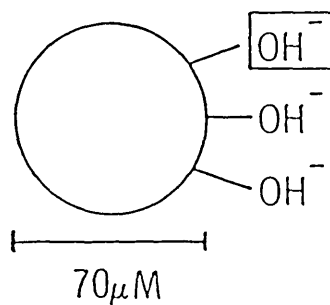
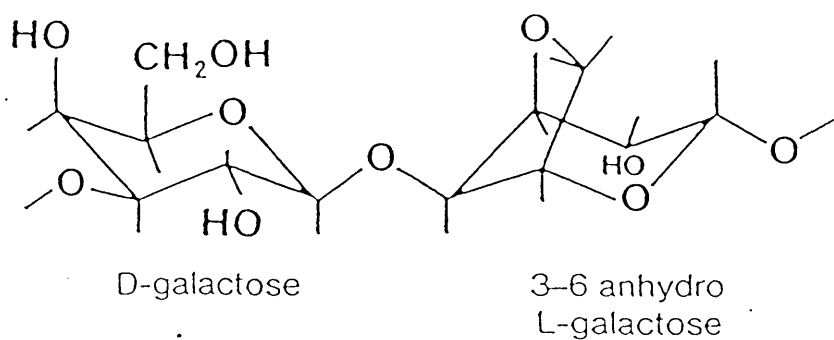


FIGURE 34 DIAGRAMATIC REPRESENTATION OF THE SEPHACRYL BEAD

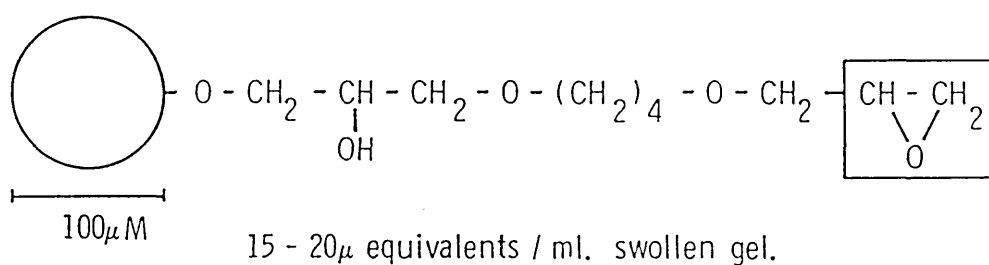
A

Agarose



B

Epoxyactivated Sepharose



C

AH Sepharose 4B

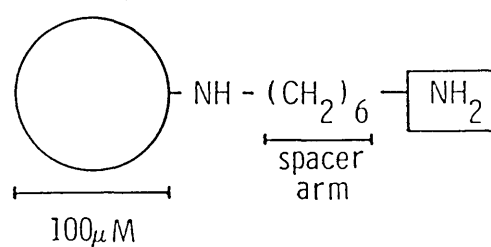


FIGURE 35

A.) THE STRUCTURE OF THE REPEATING UNIT OF AGAROSE FROM WHICH SEPHAROSE IS PREPARED

B) A DIAGRAM OF THE STRUCTURE OF EPOXYACTIVATED
• SEPHAROSE

C) A DIAGRAM OF THE STRUCTURE OF AH SEPHAROSE 4B

(REPRODUCED FROM THE PHARMACIA GEL FILTRATION BOOKLET WITH PERMISSION)

was found for each. The supports used included: Sephacryl, Sepharose resins and oligo (dN) cellulose resins (illustrated in figures 34, 35; Pharmacia, 1979; Pharmacia, 1985).

Each method of immobilisation was assessed for its efficiency in binding oligonucleotides. Quantitation of covalently coupled DNA was achieved by incorporating a trace of ^{32}P Kinase end-labelled oligonucleotide into each reaction. The extent of specific and non-specific immobilisation was determined by Cerenkov counting (Berger and Krug, 1985) the resins before and after attachment.

$$\% \text{ attached} = \frac{\text{initial dpm}}{\text{final dpm}}$$

The availability of the immobilised DNA to hybridisation was initially assessed in experiments using the enzyme S1 nuclease. The results helped determine the proportion of DNA potentially available for hybridisations, as radioactive counts lost from the solid support were a reflection of the concentration of DNA exposed to the enzyme.

3.4.1 Immobilisation by carbodiimide activation

Initially the optimum concentration of DNA for immobilisation via the 5' phosphate group, using carbodiimide, was established. The coupling strategy is outlined in figure 36. The hydroxyl-containing resin used was Sephacryl, a resin found to be ideal for the immobilisation of single stranded DNA fragments (Langdale, 1985; Nicholls et al., 1987). Using sonicated and phosphorylated calf thymus DNA and Sephacryl S-1000 it was found, that maximum coupling was achieved with an input concentration of 500 pmol DNA/g resin (or 25 pmol for 50mg resin; see figure 37). The average

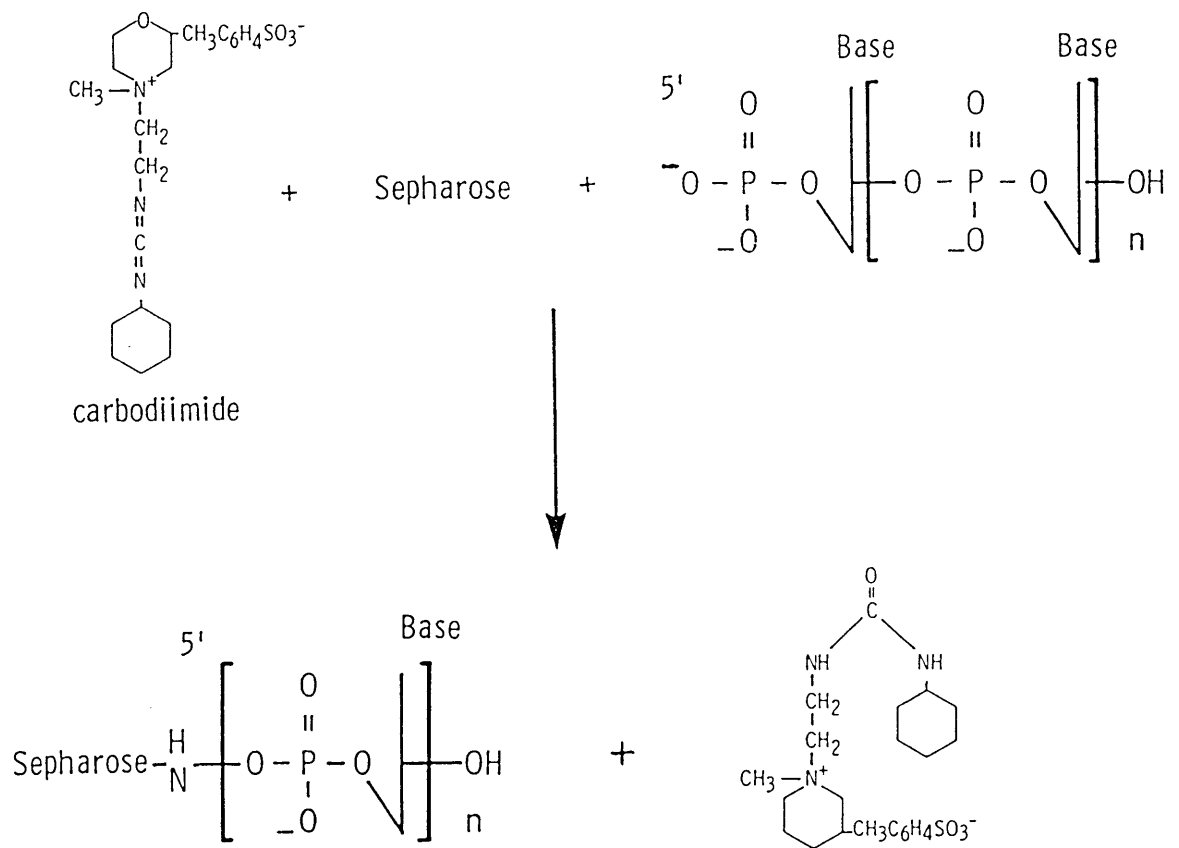


FIGURE 36 Attachment of oligonucleotides to Sepharose beads via their terminal phosphate grps. using carbodiimide.

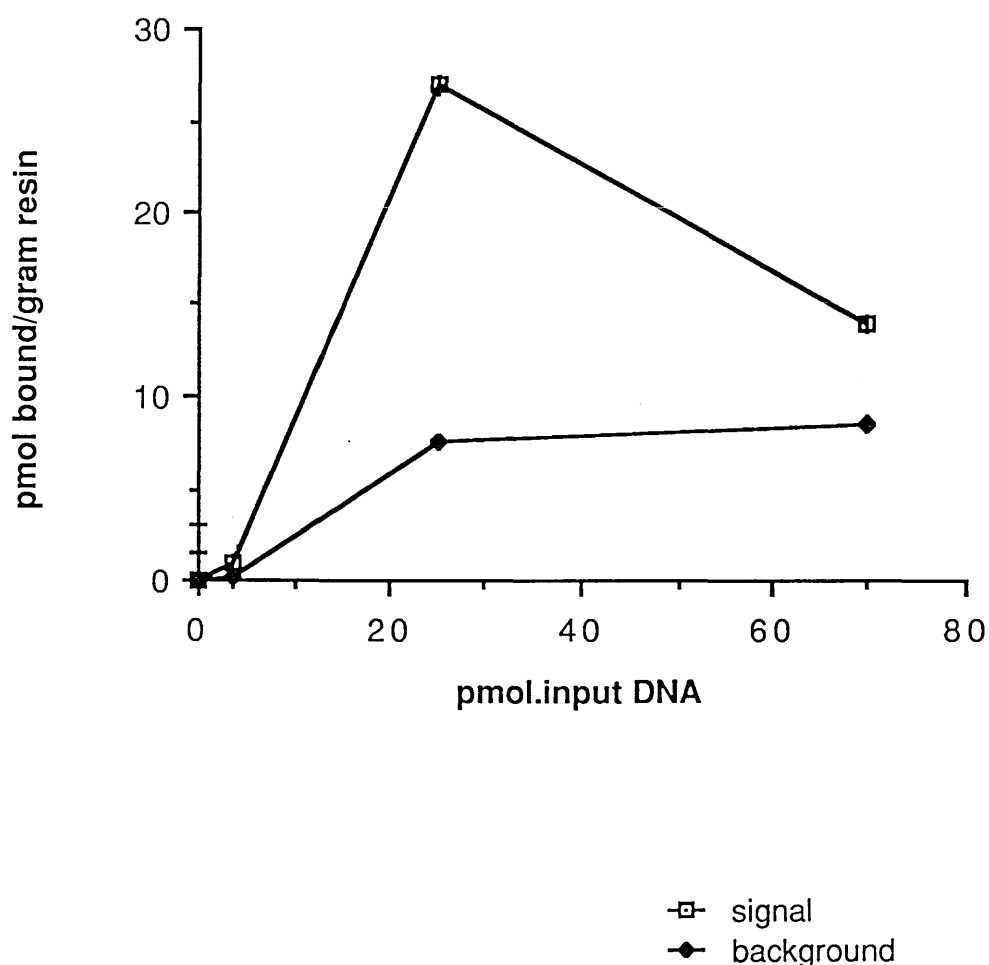


FIGURE 37 OPTIMUM CONCENTRATION OF DNA FOR CARBODIIMIDE MEDIATED IMMOBILISATION, ESTABLISHED WITH SEPHACRYL S-1000 AND CALF THYMUS DNA

The efficiency of immobilisation was determined using 3.5, 25 and 70 pmol of single-stranded calf thymus DNA sonicated to an average length of 300 kb. Each reaction was performed with 50 mg Sephacryl S-1000 resin 5% ^{32} P kinase end-labelled calf thymus DNA. Background immobilisations were determined by omitting carbodiimide from the reactions. The results were determined by Cerenkov counting the washed resins, and relating the final immobilisations with the initial input concentration of DNA.

percent input DNA immobilised was however found to be extremely low: 1-6% total immobilisation of which 0.2-1.5 % was found to consist of non-specific attachments. The results are illustrated in table 10. Input DNA concentrations above 500 pmol/g resulted in reduced coupling efficiencies.

Sepharose resins with sugar hydroxyl groups and other reactive ligands were examined for their coupling efficiencies. Data by Allfrey and Inoue (1978) found Sepharose to be a resin most suited for carbodiimide mediated immobilisations. High binding capacities with respect to the coupling of nucleoside phosphates to Sepharose was also described by Schott (1985).

Two beaded Sepharose support systems were used. They were chosen for their pore structure and for their low non-specific adsorption properties. They were also selected for their mechanical stability at temperatures of up to 100°C (Lund, 1985). One of the resins used was AH Sepharose 4B, containing a free primary amino group at the end of a 6-carbon spacer arm. Another resin was Epoxy Sepharose 6B containing long hydrophilic spacer arms with a free epoxide (oxirane) group, normally used for coupling to hydroxyl or primary amino groups. This coupling normally carried out as a spontaneous reaction at high pHs did not occur at low pHs. Neither of the two resin ligands were expected to interfere with the immobilisation reaction, only limit the available hydroxyl sites and perhaps reduce non-specific adsorption. A third resin support system used was Sephacryl S-1000.

Single stranded calf thymus DNA (300bp) and oligonucleotides (20mer, 50mer and 100mer) kinase end-labelled with dATP were immobilised to all three supports via carbodiimide immobilisation. Phosphorylation of the 5' end was found to occur with 40-60% efficiency when T4 kinase enzyme was used (data not shown). The phosphorylation of sonicated calf thymus DNA was less critical as

1. RESIN: Sephacryl S-1000

PROBE	INPUT DNA (pmol)	DNA BOUND (pmol/g resin)	%
Beta globin (20mer)	5	1.4	1.4
	25	30	6
Calf thymus (300bp)	3.5	0.91	1.3
	25	27	5.4
	70	32.2	2.3
Calf thymus control (300bp)	3.5p	0.168	0.24
	25p	7.5	1.5
	70p	8.4	0.6
Beta-globin control (20mer)	5p	6	0.6
	25p	5	1

2. Resin: AH Sepharose 4B

PROBE	INPUT DNA (pmol)	DNA BOUND (pmol/g resin)	%
Calf thymus 300bp	70p	322	23
Beta globin 20mer	25p	134	26.7
50mer	25p	108	21.6
100mer	25p	55	11.0
Beta-globin control 20mer	25p	26	5.2
50mer	25p	5	1.0
100mer	25p	28	5.6
Calf thymus control 300bp	70p	81.2	5.8

3. Resin: Epoxy activated Sepharose 6B

PROBE	INPUT DNA (pmol)	DNA BOUND (pmol/g resin)	%
Calf Thymus 300bp	70	56	4
Beta globin 20mer	25	81	16.2
Beta-globin control 20mer	25	9.5	1.9
Calf thymus control 300bp	25	6	1.2

TABLE 10 EFFICIENCY OF COVALENT COUPLING OF DNA BY CARBODIIMIDE ACTIVATION TO THREE DIFFERENT RESIN TYPES

The results listed in these tables are all the mean results of a minimum of two experiments, which have been performed using 50mg of the beaded resin support. The coupling reactions were each performed with 5% ^{32} P kinase end-labelled DNA and the efficiency of coupling was determined by Cerenkov counting the support bound isotope. Oligonucleotides were phosphorylated at the 5' ends prior to immobilisation. The results show that AH Sepharose is the resin most suited to this method of immobilisation.

sonication appeared to remove only a small proportion of the 5' phosphate groups.

The Sepharose resins provided better coupling yields to those recorded for the binding to the Sephacryl support system. The average binding was between 180-322 pmol/gram, for the AH Sepharose 4B resin and between 50-80 pmol/gram for the Epoxy Sepharose resin. The non-specific adsorption (background binding) was approximately 19% for the first resin and 13% for the second. The average results are listed in table 10.

AH Sepharose 4B, which appeared to be most suited for this coupling strategy was also used to compare the immobilisation efficiencies of oligonucleotides of different nucleotide lengths. It was found that longer sequences hybridised with lower efficiencies than short sequences. This is illustrated in figure 39. Furthermore nuclease digestion, with S1 nuclease, revealed that 60-77% of the oligonucleotides, immobilised to the AH Sepharose 4B resin, were covalently linked and available to hybridisation. A slightly greater concentration of 100mer was digested with S1 nuclease than 20mer, however this may have been due to easier binding of the enzyme to the 100mer.

To establish the stability of the covalent bond between resin and oligonucleotide mock hybridisations were set up. These exposed the resins to the manipulations and temperatures required of a sandwich assay. The experiments were set up with 3' ³²P end-labelled oligonucleotide resins, to determine whether DNA was being lost from the resins through mechanical shearing or whether a general loss characteristic of carbodiimide immobilisation was occurring. The results in table 13. show that approximately 10-20% of the immobilised DNA was lost purely as a result of exposing the resins to higher temperatures. The release did not appear to be caused by DNA

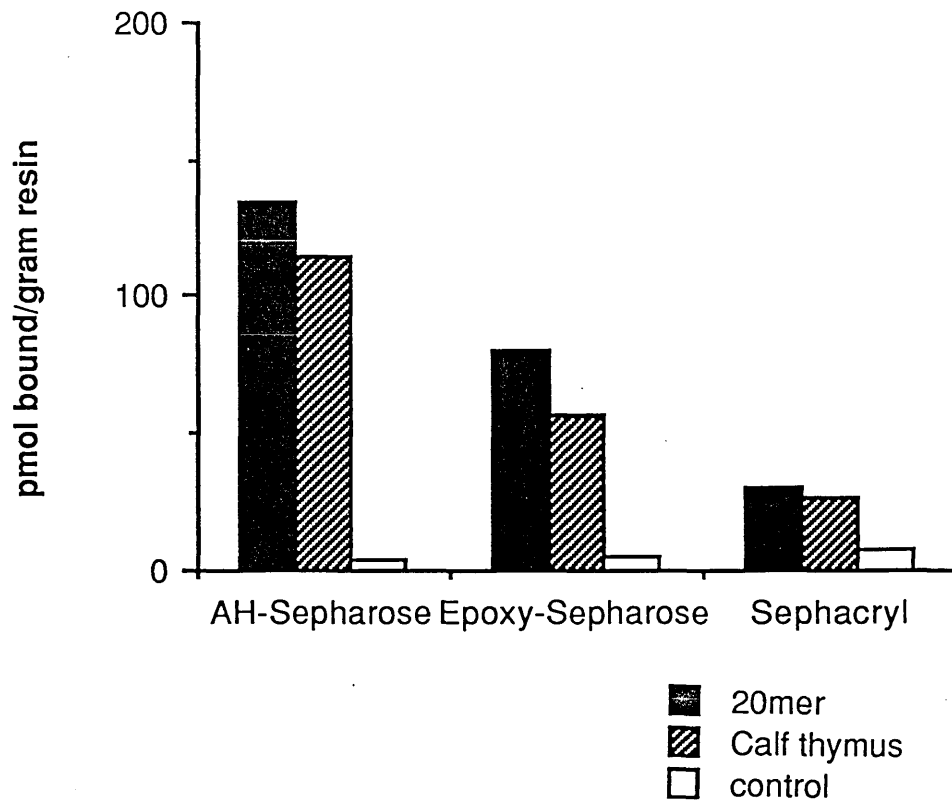


FIGURE 38 THE AVERAGE COUPLING EFFICIENCIES FOUND FOR CARBODIIMIDE MEDIATED IMMOBILISATION USING THREE RESIN TYPES.

The results shown in this bar graph are the mean immobilisation efficiencies found from four experiments using carbodiimide and the three resin support systems. The coupling reactions were each performed with 25 pmol phosphorylated, single-stranded DNAs and 50 mg of the beaded support. The results show that AH Sepharose is the resin most suited to this method of immobilisation.

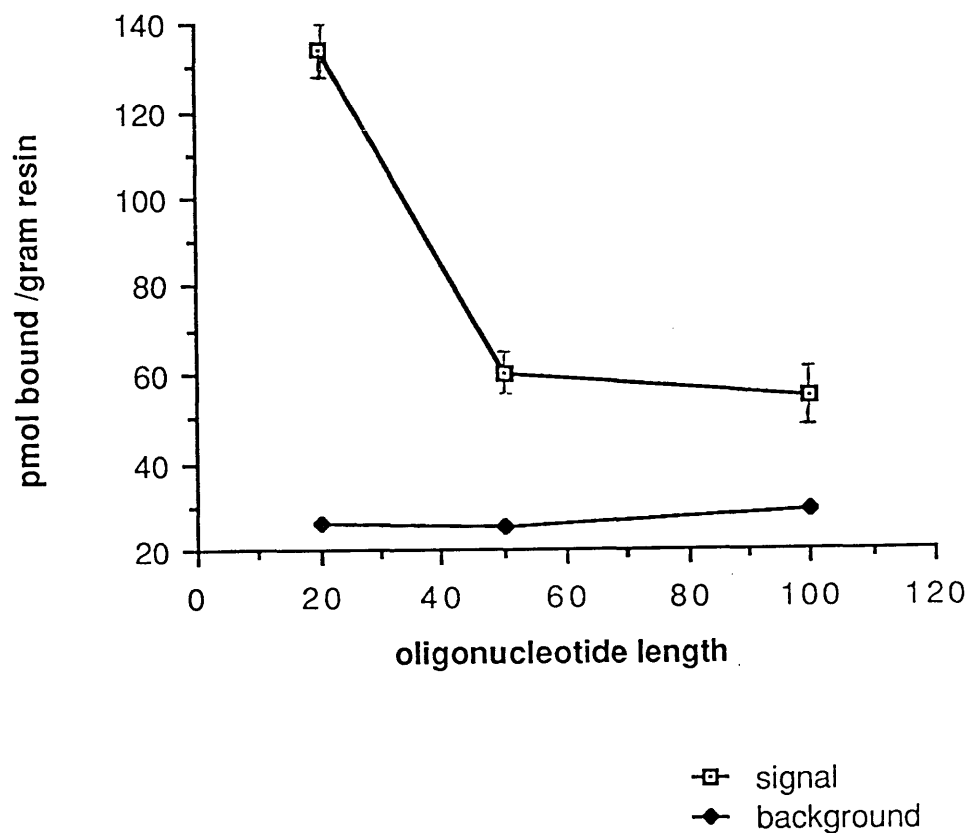


FIGURE 39 OPTIMUM OLIGONUCLEOTIDE LENGTH ESTABLISHED FOR CARBODIIMIDE MEDIATED IMMOBILISATION, USING 50 MGS AH Sepharose 4B RESIN AND 25 PMOL BETA-GLOBIN SPECIFIC OLIGONUCLEOTIDE.

The results show that short oligonucleotides are immobilised more efficiently than longer sequences. Non-specific oligonucleotide attachments appear to be independent of length. The error bars relate to the maximum and minimum percentage deviations recorded around the mean for these immobilisations.

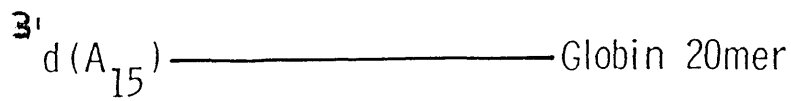
damage, since resin exposed only to the elevated temperatures and no harsh washing procedures displayed an equal loss. DNA loss was found to be proportional to nucleotide length oligonucleotide. A greater proportion of 100mer was lost from the resins than 20mer oligonucleotide. Exactly why this occurred was unclear.

3.4.2 Enzymatic immobilisation

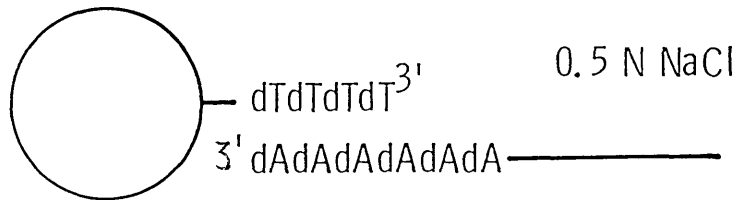
The 20mer and 50mer oligonucleotides covalently attached to the cellulose resins, using this enzymic coupling method, coded for the sense strand of the beta-globin gene. As coupling relied on the ligation of phosphate groups to the 3' hydroxyl groups of the oligo (dT) chains, sequences were phosphorylated at the 5' ends. A complementary dA tailed 35mer oligonucleotide was used as the "linker" sequence, aligning the probes for immobilisation to the 3' ends of the mononucleotide chains of the cellulose resins. The coupling strategy is outlined in figure 40.

Initial immobilisation experiments were performed with different concentrations of oligonucleotides, for covalent attachment to oligo (dT) cellulose resin. A linear relationship between the input concentration of DNA and the efficiency of immobilisation was found. Twice as much oligonucleotide was immobilised when 10 pmol instead of 5 pmol was added to the reaction. The results are shown in table 11. It was also found that the ligation reaction between the 5' end of the oligonucleotide and the 3' end of the oligo (dT) chain, was an important limiting step in the efficiency of immobilisation. Generally coupling yields were limited by the efficiency of the T4 ligase enzyme and not the Klenow enzyme, used to fill in the sequence gaps between oligonucleotide and resin.

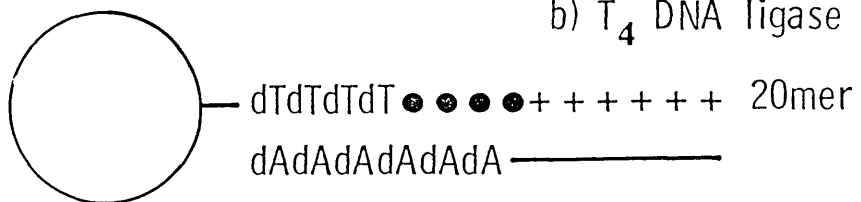
Oligo dT cellulose (8-12 base chain length) had an approximate



I. Hybridization



II. Gap filling and ligation



III. Strand Separation

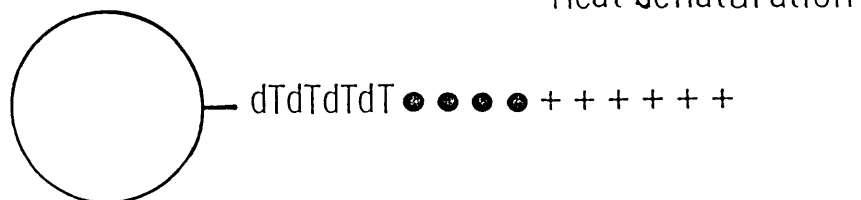


FIGURE 40 DIAGRAMATIC REPRESENTATION OF A METHOD OF ENZYMIC IMMOBILISATION USED TO LINK OLIGODEOXYRIBONUCLEOTIDES VIA THE 5' PHOSPHATE GROUPS TO OLIGO (dT) CELLULOSE

(Adapted from the methods by Goldkorn and Prockop, 1986)

Sample	% Hybridisation	% Ligation	% Strand separation	pmol/gram immobilised
(d A) tailed 20mer (linker)	18	14	1.95	100
complementary 20 mer + (dA)	37	30	20	2000 (a)
tailed 20mer (linker)	19	17.5	13	650 (b)

TABLE 11 ENZYMIC IMMOBILISATION LINKING OLIGONUCLEOTIDES TO
AN OLIGO (dT) CELLULOSE SUPPORT MATRIX

The poly (dT) cellulose resin (type 7) used had an average chain length of 8-12bp (with lengths up to twenty-five nucleotides) and was available to binding 80-100 A 260_m units of poly (A) per gram of resin. The results reflect the typical DNA binding efficiencies encountered at each step with a starting concentration of 5pmol (dA) tailed "linker" oligonucleotide and b) 5 pmol or a) 10pmol complementary 20mer oligonucleotide for immobilisation. Oligonucleotide tracers were added at each step in order to follow the efficiency of immobilisation. The results show that a greater percentage of the complementary oligonucleotide is immobilised (35%) when an excess is added to the reaction.

40 mg T residues per gram of cellulose resin and a potential binding capacity of 6 μ mol dA tailed oligonucleotide. The coupling results obtained using this resin suggested that only 6-12 % of the total binding sites available were used for oligonucleotide immobilisation. The average binding efficiencies were observed as 1200 pmol/gram cellulose resin. Non-specific binding to the resin was low, between 3-5%, equivalent to approximately 400 pmol/gram resin.

When complementary 50mer oligonucleotide was immobilised to the same resin a 27% drop in binding efficiencies was recorded. This drop in binding from 1200 pmol/gram resin to 880 pmol/gram resin was not reflected in the amount of non-specific adsorption which remained the same.

A comparison between the binding efficiencies of three different cellulose resins was made;

- a) oligo dT cellulose (average chain length of 10 bases)
- b) oligo dT cellulose (average chain length of 15 bases)
- c) oligo dU cellulose (average chain length of 10 bases)

The oligonucleotide used for this comparison was the 50mer beta-globin sequence. Figure 41 illustrates the 42% drop in the binding efficiency recorded (645 pmol/gram resin), when the shorter chained oligo dT cellulose resin was used. The oligo dU resin on the other hand revealed similar coupling efficiencies to the long chained oligo (dT) cellulose, suggesting that the uridine moieties formed a more stable hybrid complex with the dA tailed "linker" oligonucleotide than thymine moieties. Non-specific adsorption was similar for all three resins, between 3-6%.

These results compared reasonably well with the results, obtained by Goldkorn and Prockop (1986) using enzymatic procedures for the immobilisation of restriction fragments. Unlike restriction fragments however, the efficiency of covalent oligonucleotide linkage

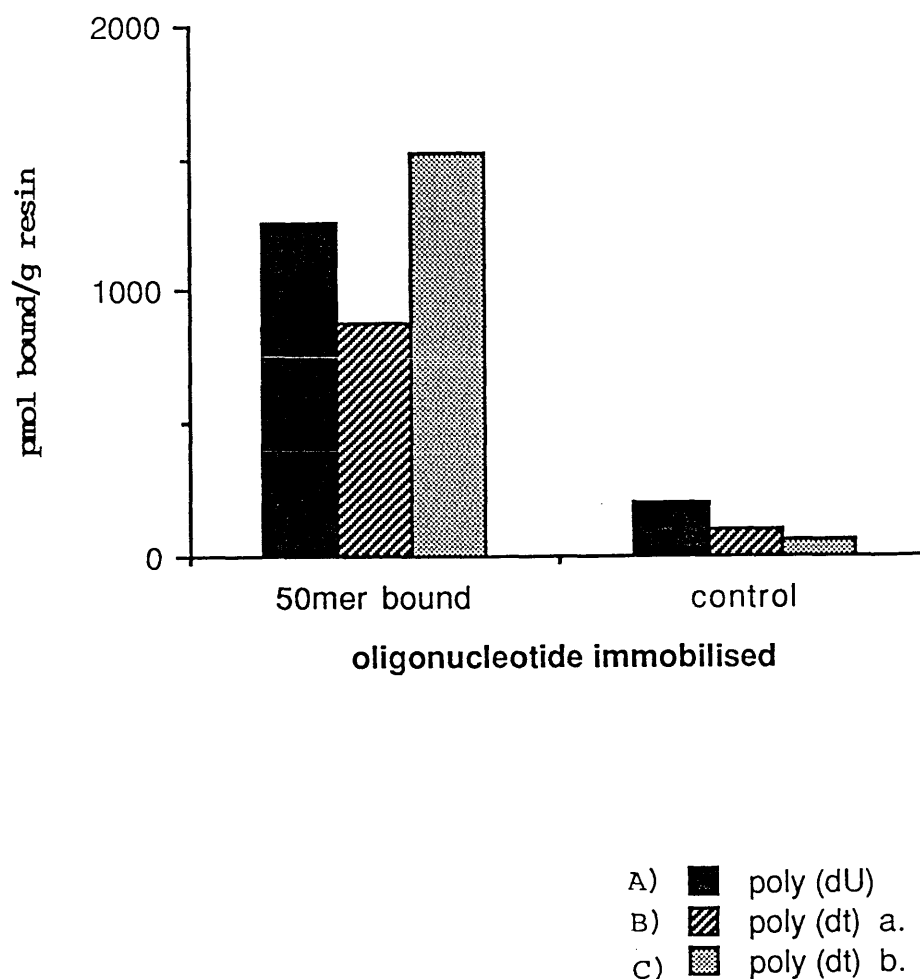


FIGURE 41 THE AVERAGE ENZYMIC LINKAGE RECORDED FOR THE IMMOBILISATION OF OLIGONUCLEOTIDES TO CELLULOSE RESINS

The oligonucleotide chain length of the cellulose resins used were as follows: A) and B) an average of 8-12 nucleotides and C) an average of 12-18 nucleotides. All immobilisations were performed with 10 pmol of complementary oligonucleotide DNA and 5pmol of (dA) tailed "linker" DNA along with 10mg of the respective cellulose resin. The results show that the cellulose resin with the longer chain length enables marginally higher coupling efficiencies. Also apparent is that a slight increase hybrid stability of the (dA) tailed linker oligonucleotide is probably achieved through U:A hydrogen bonding.

relied on successful phosphorylation of the oligonucleotide to be immobilised. The results obtained were therefore probably an underestimate of the true binding efficiencies, suggesting that oligonucleotides might even be more suited to enzymatic immobilisation, than restriction fragments.

Nuclease digestion experiments were not carried out with these resins. No apparent loss of covalently bound oligonucleotide from the cellulose resins was recorded at elevated temperatures.

3.4.3 Immobilisation by diazotization

With the knowledge that immobilisation by diazotization relied on the coupling of free base amino groups (see figure 42), experiments were initially designed to determine the effect oligonucleotide length had on binding efficiencies. Using Sephacryl S-1000 as a support matrix three oligonucleotide lengths all coding for the beta-globin gene were immobilised: a) 20mer (s), b) 50 mer (s) and c) 100mer (as). The optimum DNA concentration of 140 pmol/g resin, found by Langdale and Malcolm (1985) was used in these experiments.

The immobilisation results revealed that oligonucleotide length was in fact critical for stable covalent linkage. Oligonucleotides with only 20 nucleotides were less efficiently immobilised (10 %) than oligonucleotides with 100 nucleotides (54 %). The amount of non-specific DNA attachments (background binding) on the other hand was less dependent of length. This is illustrated in figure 43. When sonicated calf thymus DNA was immobilised to the same resin, the immobilisation achieved (57.5 pmol/gram resin) was similar to the yields obtained for the 100mer oligonucleotide (75.6 pmol/gram resin). This suggested that within a hundred bases enough coupling events had occurred for stable binding.

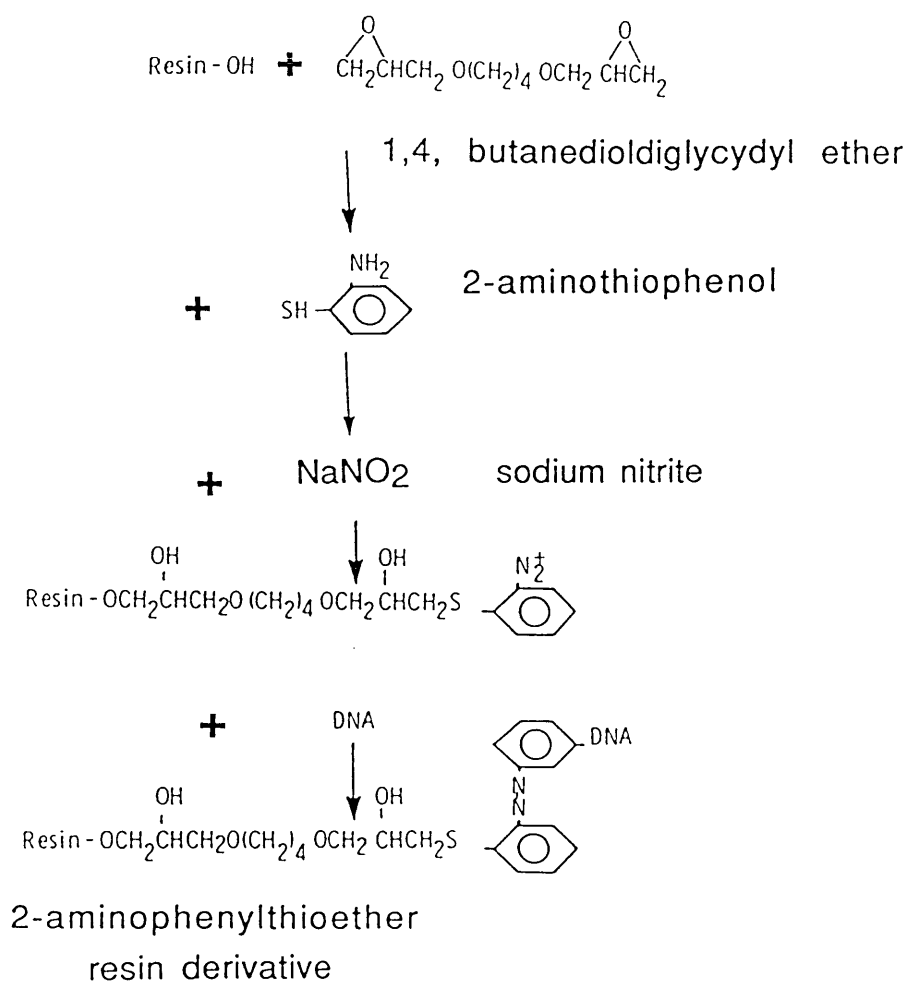


FIGURE 42 THE CHEMISTRY INVOLVED IN THE COVALENT IMMOBILISATION OF DNA VIA DIAZOTIZATION TO FREE HYDROXYL GROUP CONTAINING SUPPORT SYSTEMS

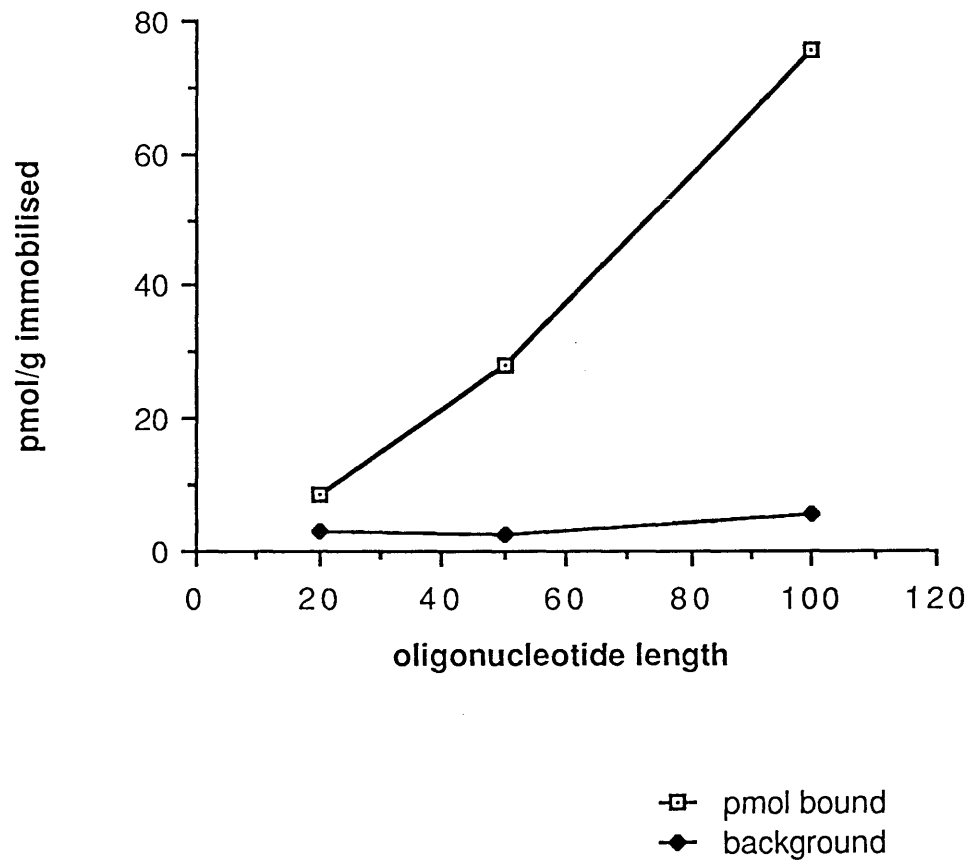


FIGURE 43 IMMOBILISATION OF OLIGONUCLEOTIDES VIA DIAZOTIZATION TO SEPHACRYL S-1000

The immobilisation reactions were performed using 140 pmol oligonucleotide per gram of Sephacryl S-1000 resin support, as described in section 2.3.13.3 of the methods. The oligonucleotides immobilised were all beta-globin specific sequences, coding for the region around the sickle mutation site or immediately upstream of this site, in the case of the 100mer.

The overall binding efficiencies to the solid support through diazotization recorded were low, but comparable to previous data obtained for restriction fragment immobilisation to Sephacryl S-1000 (Bunemann et al 1982). It is interesting to note that the binding efficiencies obtained for 20mer immobilisation via diazotization were similar to those efficiencies recorded for carbodiimide mediated end-linkage. The amount of non-specific binding was also comparable.

Nuclease digestion with S1 nuclease released approximately 60-70% of the non-covalently bound 100mer oligonucleotide from the resin (see table 17). This again was comparable to results obtained by Langdale and Malcolm (1985). Furthermore an average loss of 20 % immobilised oligonucleotide was detected in the control reactions. DNA loss was also recorded by Bunemann et al (1982,a)) in similar reactions. He described this loss as characteristic of the biphasic manner in which DNA was immobilised by diazotization. After an initial loss of up to 50% at elevated temperatures the DNA materials behaved in a more stable manner with a daily loss of 0.5-1%.

DNA losses observed upon exposure of the 100mer oligonucleotide resins to conditions simulating those of a sandwich hybridisation assay, were found to be around 17%. Similar losses were recorded for resins containing immobilised calf thymus DNA.

3.4.4 Summary

1. Carbodiimide mediated immobilisation was suited for resins with sugar hydroxyl groups such as AH Sepharose 4B. The amount of DNA attached was between 180-320 pmol/g resin, of which 6-11% was not covalently attached.
2. Carbodiimide immobilisation of oligonucleotides appeared to be slightly length dependent, with 40-60% of the DNA immobilised

available to hybridisation.

3. Enzymic immobilisation was suited for either long chained oligo (dT) cellulose resins or oligo (dU) resins. The potential binding capacity of these resins was very high and non-specific binding sites were low in number. Oligonucleotides could be immobilised at concentrations of 1-1.5 $\mu\text{mol/gram}$ resin, of which 5% consisted of non-specific bindings.
4. A slight difference in the enzymatic immobilisation of long and short oligonucleotides was detected. A 27% drop in coupling yields was recorded for the immobilisation of 50mers when compared to the immobilisation of 20mers.
5. Both carbodiimide and enzyme mediated immobilisation yields were limited by the efficiency of 5' end phosphorylation with the enzyme T4 kinase.
6. Oligonucleotide immobilisation by diazotization was clearly length dependent. Of the initial input DNA, 40-50% more was immobilised for oligonucleotides with 100 bases than there was to those of 20 bases.
7. Of the DNA immobilised 30-50% was available for hybridisation.
8. An initial loss of 10-30% DNA immobilised by diazotization was found, when the resins were subjected to raised temperatures. A similar but smaller loss was also recorded for DNA immobilised by carbodiimide activation. No apparent DNA loss was found associated with the enzymatic immobilisation.

3.5 One-step hybridisations

To find the immobilisation strategy best suited for a sandwich hybridisation assay, the oligonucleotide resins were initially tested for their ability to capture target DNA. This was done through one-step hybridisations with Kinase end-labelled beta-globin recombinant DNA fragments. Both the normal (Hbf-5) and the sickle (Hbl-S) recombinant genes were used as target DNAs. The gene inserts were isolated from the plasmid DNA through restriction digestion. Some of the DNA was restricted with the enzyme Hae III to generate the 208bp fragment around the sickle cell mutation site. The effect of target length as well as the constraints imposed on hybridisation by the immobilised oligonucleotides were established. The amount of immobilised oligonucleotide used in the hybridisation reactions, was determined from the initial coupling yields. The optimum concentration of target DNA, the optimum hybridisation time, temperature and the washing procedures required to remove non-specific binding were found. For all experiments the hybridisation efficiencies were calculated as follows:

$$\frac{\text{dpm of hybridised target or dpm of nonspecific hybridisations}}{\text{dpm of initial input target DNA}} \times 100$$

The non-specific attachments were determined through hybridisation experiments with non-complementary target DNA sequences and with control resin samples (without the immobilised capture oligonucleotide).

3.5.1 One-step hybridisations to DNA immobilised with carbodiimide

Stringent hybridisations with different lengths of target DNA were performed using resins with both immobilised oligonucleotides and with immobilised calf thymus DNA. The hybridisation temperatures were chosen to suit hybridisation of the capture probes. For the 20mer globin oligonucleotide, hybridisations were carried out at 50°C, and for capture probe lengths of more than 50 base pairs, the temperature was set at 65°C. Upon hybridisation the resins were washed as described in the materials and methods section. Non-specific hybridisations were determined with non-complementary DNA sequences, either 20mer oligonucleotides or 1.6 kbp recombinant alpha-1-antitrypsin DNA.

Hybridisations with 0.5 pmol of the oligonucleotide immobilised resins, were performed with 10 fmol complementary 208bp or 1900bp beta-globin target DNA. A 50 fold excess oligonucleotide capture probe was used in the reactions. This excess had been found to be the optimum concentration, for the maximum hybridisation efficiency, using recombinant DNA fragments (Langdale and Malcolm, 1985). All three oligonucleotide resins displayed the same availability to hybridisation. All three resins also displayed the same amount of non-specific hybridisation. The results depicted in table 12, indicate however, that hybridisation of the 1900bp target fragment was less efficient than that of the 208bp fragment. This difference could be explained if the shorter target length hybridised with greater stability than the longer target. Strand displacement and reannealing of the target DNA was perhaps occurring more readily with long DNA fragments than with shorter fragments.

In an attempt to improve hybridisation efficiencies, hybridisations under the same conditions with 50mer and 100mer oligonucleotides resins and 208bp beta-globin target DNA were set

<u>Immobilised DNA</u> (capture probe)	<u>Target</u> <u>length</u>	AH Sepharose	Epoxysepharose	Sephacryl
20mer beta-globin				
	1.9 kb	11 $\pm$ 5	12 $\pm$ 6	11 $\pm$ 3
	208 bp	23 $\pm$ 5	16 $\pm$ 8	--
control		1-2	1-2	1-2
Calf thymus (300bp)	300 bp	7 $\pm$ 5	7 $\pm$ 4	12 $\pm$ 5

TABLE 12 ONE-STEP HYBRIDISATION EFFICIENCIES OF 500 FMOL CARBODIIMIDE IMMOBILISED OLIGONUCLEOTIDE TO 10 FMOL OF FREE TARGET DNA EXPRESSED AS A PERCENTAGE.

The results are the average results of at least four hybridisation experiments which were all performed under stringent hybridisation conditions as described in section 2.3.15 of the methods. The $\pm$ values are the maximum and minimum percentage deviations about the mean found for each hybridisation reaction. The limits of detection were found to be around 1 fmol (data not shown). The average weights of resin present in each reaction were 1.5-2 mg for AH Sepharose 4B, 9-10 mg for the Epoxy-activated Sepharose and 3-5 mg for the Sephacryl resin.

Immobilised DNA (capture probe)	% One-step hybridisation		Immobilised DNA	
	2nd wash	60°C wash	lost during hybridisation manipulations (%)	availability to S1 digestion (%)
20mer	20.8	12	7 $\pm$ 5	60 $\pm$ 8
50mer	20.5	11	20 $\pm$ 10	65 $\pm$ 12
100mer	17	9	47 $\pm$ 24	77 $\pm$ 10
<u>control:</u>				
resin	3.2	2	--	--
target	6	3	--	--

TABLE 13 THE DNA LOSSES ASSOCIATED WITH ONE-STEP HYBRIDISATIONS, AND THE AVAILABILITY TO HYBRIDISATION USING OLIGONUCLEOTIDE CAPTURE PROBES IMMOBILISED VIA CARBODIIMIDE ACTIVATION

The percentage values are derived from the mean results of at least two sets of (duplicate) experiments. The resin used for oligonucleotide immobilisation was AH sepharose 4B. Each hybridisation reaction was performed with 250 fmol capture probe and 5 fmol labelled target DNA. The sequences were all globin specific and the target DNA was a 208 bp fragment coding for the sequence of DNA around the sickle mutation site. The immobilised DNA lost during hybridisation manipulations could either be the result of sheering effects or leakage of the covalently bound oligonucleotide from the support. The % DNA loss seen upon S1 digestion is a reflection of the oligonucleotide sequence available to hybridisation once immobilised by carbodiimide activation. The +/- values relate to the maximum and minimum percentage losses recorded.

up. Increasing the capture probe lengths to equal those of the target DNA had little effect on the observed hybridisation efficiencies.

When sonicated calf thymus DNA, immobilised to the three resins, was hybridised to single stranded complementary DNA, even lower hybridisation efficiencies were observed. This result depicted in table 12, was somewhat surprising, as the DNA lengths of both capture probe and target length were of equal size. Another factor must have been interfering with the hybridisation reaction, such as reannealing of the immobilised strands.

In all of these experiments using carbodiimide linked DNA resins, an inexplicable instability of the hybrid complex was observed, whether the sequences were equal in length or not. Vigorous washing procedures quickly removed specific hybrid complexes (table 13). The DNA loss could not be entirely explained by the normal loss upon exposure to higher temperatures. The loss in specific hybridisation could also have been attributed to other causes. Perhaps end immobilisation interfered with normal duplex formation creating less stable hybrid complexes.

The detection limit of this one-step hybridisation assay, using Kinase end-labelled target DNA was 1-10 fmol. It appeared that this might not suit a sandwich hybridisation system.

3.5.2 Hybridisations of enzymatically immobilised DNA

The ability of the immobilised DNA to hybridise to its complementary sequence was initially determined using ^{32} P end-labelled 20mer oligonucleotides 200bp and 1900bp target fragments. Two oligonucleotide lengths coding for the beta-globin gene around the site of the sickle mutation were immobilised to oligo (dt) cellulose: a 20mer and a 50mer sequence. Non-specific hybridisations

were determined with a non-complementary 20mer sequence and a 1600bp restriction fragment.

A 50 fold excess immobilised oligonucleotide resin was used in hybridisation experiments with 1 fmol target DNA. From table 14, it can be seen that target lengths closer in length to the capture probe hybridised with greater efficiency than longer sequences. Oligonucleotides hybridised with greater efficiency to 20mer and 50mer capture probes than DNA restriction fragments. In all experimental hybridisations the non-specific bindings were between 4-8%. The hybridisation results using restriction fragments were approximately half of the 45-51% efficiencies detected by Goldkorn and Prockop (1986), where the immobilised capture DNA's were restriction fragments.

Differential hybridisation was found when oligonucleotides with a single base mismatch were hybridised to immobilised complementary sequences. No significant differential hybridisation occurred between the immobilised oligonucleotides and DNA restriction fragments. The lack of differential hybridisation using restriction fragments suggested that the spacer-arm was providing binding sites for the DNA .

Hybridisations with a constant amount of immobilised 50mer resin (0.2 pmol) and with increasing concentrations of 1900bp target DNA, from 1-40 fmol, were carried out to find the optimum immobilised DNA:free DNA ratio. The results suggested that the percentage hybridisations were greatest, when the resin was present in 50-100 fold excess. The data obtained is illustrated in figure 44. As the target DNA concentration was increased, an overall increase in signal was obtained. When the target concentrations were decreased, an increasing proportion of the signal obtained was due to nonspecific attachments.

Oligonucleotide immobilised (50 fmol DNA)	Free target DNA (1 fmol DNA)	% Hybridisation
20mer globin (s)	20mer globin (normal)	33 $\pm$ 7
	20mer globin (sickle)	21 $\pm$ 6
	1900bp globin (normal)	22 $\pm$ 4
	1900bp globin (sickle)	20 $\pm$ 6
Control	20mer	4 $\pm$ 2
	1600bp	8 $\pm$ 4
.....		
50mer globin (s)	20mer globin (normal)	30 $\pm$ 6
	20mer globin (sickle)	14 $\pm$ 4
	208bp globin (normal)	a) 26 $\pm$ 8 b) 12 $\pm$ 6 c) 32 $\pm$ 10
	1900bp globin (normal)	21 $\pm$ 5
	1900bp globin (sickle)	20 $\pm$ 4
Control	20mer	4 $\pm$ 1
	1600bp	8 $\pm$ 5

TABLE 14 ONE-STEP HYBRIDISATIONS WITH OLIGO (dT) CELLULOSE IMMOBILISED OLIGONUCLEOTIDES AND WITH SINGLE STRANDED, FREE TARGET DNAS

The one-step hybridisations were performed with 50 fmol beta-globin specific oligonucleotide acting as the immobilised capture probe and 1 fmol of 32 P kinase end-label target DNA. The target sequences were both complementary oligonucleotide and restriction fragments of the recombinant plasmids coding for the beta-globin gene. The hybridisations were carried out as described in section 2.3.15 of the methods. The hybridisation efficiencies recorded as a, b, and c were performed using the following cellulose support systems: a) Oligo (dT) cellulose resin with a poly dT chain length of 8-12 bp.; b) with a poly dU chain length of 8-12 bp.; c) with a poly dT chain length of 12-18 bp. Differential hybridisation between the normal and sickle genotypes was achieved with oligonucleotide target DNA's.

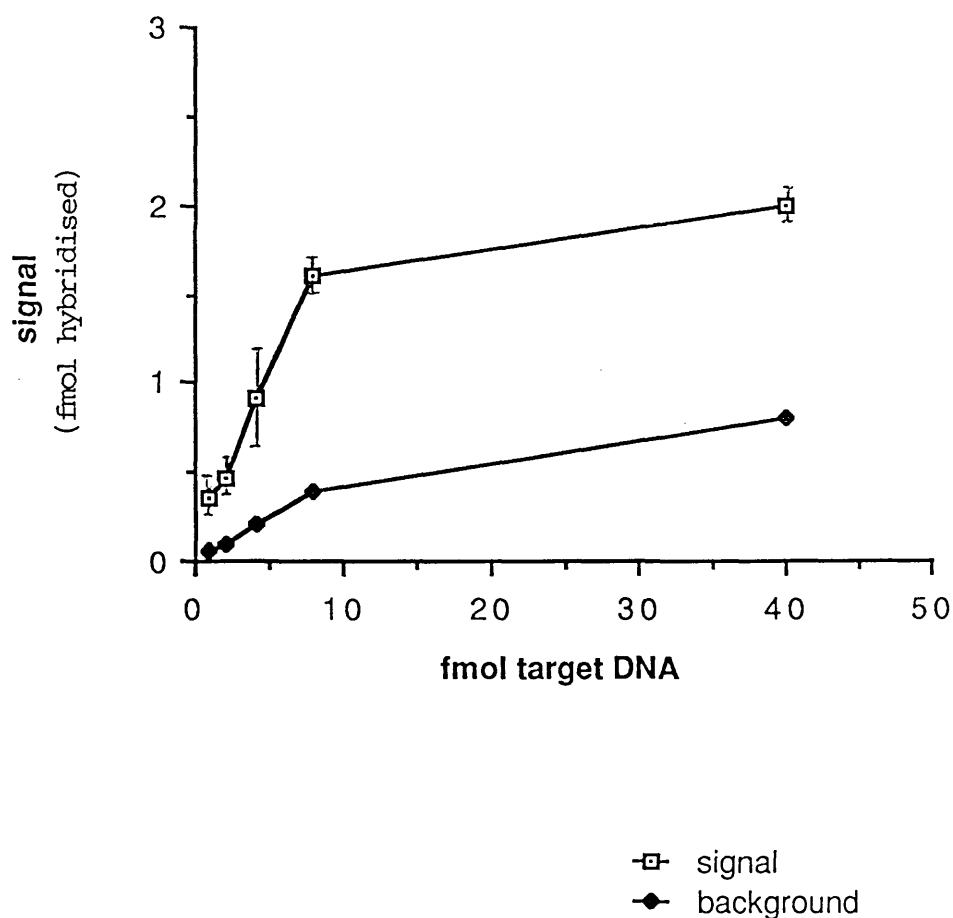


FIGURE 44 ONE-STEP HYBRIDISATION WITH INCREASING TARGET CONCENTRATIONS USING THE BETA-GLOBIN RECOMBINANT DNA INSERT

The cellulose resin immobilised oligonucleotide was 50bp in length and the concentration of the "capture probe" was 200 fmol (0.4 mg resin). The cellulose resin used had an original chain length of 8-12 (dT) nucleotides. The 1900bp target DNA of Hbf-5 was ^{32} P kinase end-labelled and was used in the hybridisation reactions at concentrations of 40, 8, 4, 2 and 1 fmol. Background signals were determined through hybridisations with non-complementary target DNA (1600bp in length). The error bars show the maximum and minimum deviations about the mean hybridisation efficiencies recorded for each target DNA concentration.

Through a series of time course experiments, using 200 fmol immobilised 50mer oligonucleotide and 1.5 and 4 fmol 1900bp target DNA, it was found that half the maximum hybridisation ($t_{1/2}$) was probably achieved in less than 1 minute respectively (figure 45). This kinetic behaviour appeared to be similar to that described by Wetmur and Davidson (1968) for single stranded probes in mixed phase hybridisations. They found that where the free DNA was not limiting to the hybridisation, and in excess to the immobilised DNA, the kinetic rate of hybridisation, described by $t_{1/2}$, was equal to:

$$t_{1/2} = \frac{N \ln 2}{3.5 \times 10^5 L C}$$

where N= complexity of DNA

L= length of DNA

C= concentration of DNA in g/liter

Using this equation the $t_{1/2}$ values would both have been reached in a few seconds respectively. Although the exact $t_{1/2}$ values could not be measured, inaccessibility of the target DNA to the capture DNA and limiting concentrations of target DNA, in the one-step assay would probably have resulted in greater $t_{1/2}$ values, to those estimated by the equation.

The limits of detection, 1-10 fmol, were equal to those found through one-step hybridisations with carbodiimide immobilised DNA. Capture probes used at concentrations of less than 1 fmol resulted in hybridisation signals too small for significant differentiation from the background. The reasons for this limit were two fold: 1) There was a limit to the minimum amount of resin one could use. Using less than 0.07 mg resin in order to reduce the

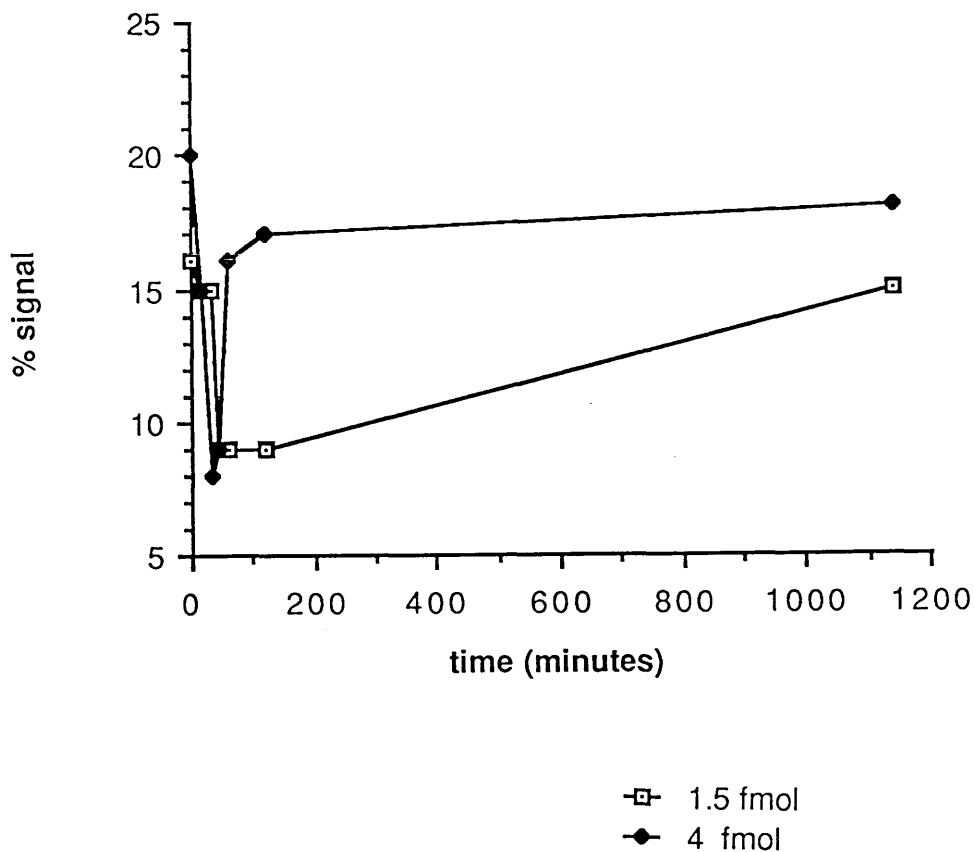


FIGURE 45 TIME COURSES DISPLAYING THE ONE-STEP HYBRIDISATION RATES WITH TWO DIFFERENT CONCENTRATIONS OF TARGET DNA USING THE 1900 bp INSERT OF THE Hbf-5 RECOMBINANT PLASMID

Using 200 fmol of the 50mer (beta-globin specific) cellulose resin, time course experiments were performed with 1.5 fmol and 4 fmol ^{32}P kinase end-labelled 1900 bp target DNA, isolated from the Hbf-5 recombinant plasmid through restriction digestion. The capture probe concentration was thus in 133 and 50 fold excess over the free target DNA. To measure the hybridisation occurring at time zero, the hybridisation components were added to one another on ice and were immediately removed through post-hybridisation washing manipulations. The graph displays the percentage hybridisations recorded for each concentration of target DNA minus the background attachments. The results show that an accurate $t_{1/2}$ value could not be measured, as this point is reached in less than 1 minute (the time taken to remove and wash away the non-specifically bound target DNA from the time zero reaction).

background effect, made manipulations more difficult and less reliable. 2) The specific activity of the radioactively labelled target DNA was limiting to the sensitivity of the reaction. There was also a limit to the counts that could be reliably detected by Cerenkov counting, using the LKB scintillation counter. A reliable signal was taken to be one at least 2 times over the background counts of the LKB counter, averaging at 125 dpm.

Comparing the enzymatically immobilised DNA hybridisations with the carbodiimide immobilised DNA hybridisations, no significant difference in efficiencies, using long target DNA fragments, was found. A noticeable difference was however found for the one-step hybridisation efficiencies using oligonucleotide target DNAs. The presence of a spacer-arm appeared to make the oligonucleotide capture probe more available to hybridisation.

Hybrid stability was however not increased by the presence of this spacer-arm. Multiple washing manipulations could also destabilise a considerable amount of the hybrid duplexes (data not shown). Despite leaving all the bases available to hybridisation, end-immobilised oligonucleotides appeared to be susceptible to destabilising factors, under stringent hybridisation conditions.

Increasing the length of the capture probe might have reduced some of this instability and increased the hybridisation efficiencies to those found by Goldkorn and Prockop (1986), of around 45-50%.

3.5.3 Hybridisations to DNA immobilised by diazotization

The parameters affecting hybridisation were also found for oligonucleotides immobilised by diazotization. Using 50-100 times excess 20, 50 and 100mer oligonucleotide resins, hybridisations with free target DNAs were initially carried out, to establish the effect

Immobilised oligonucleotide "capture probe"		Target DNA /Optimum hybridisation temperature (degrees Celsius)		
length	G / C content %	20bp	200bp	1900bp
20mer	30	30	--	---
50mer	34	20-30	45	---
100mer	19		65	65

TABLE 15 OPTIMUM HYBRIDISATION TEMPERATURE FOR ONE-STEP HYBRIDISATIONS USING OLIGONUCLEOTIDES IMMOBILISED BY DIAZOTIZATION.

The results were obtained from simple one-step hybridisation reactions with different target DNA lengths carried out at a range of temperatures. The Sephacryl immobilised capture probe was used in fifty fold excess over the target DNA concentration. All the sequences were beta-globin specific and coded for the normal genotype. The 20mer target was the complementary sequence coding for the anti-sense strand of the beta-globin gene. The results show a decrease in optimum hybridisation temperature of the hybrid complexes, where one of the strands in the duplex is immobilised by it's base amino groups. This effect is observed particularly in immobilised sequences under 100 nucleotides in length.

random base immobilisation had on duplex formation. For 20mer and 50mer capture probes, the optimum hybridisation temperatures were found to be 10-20°C lower than expected. The optimum temperature for the hybridisation of the 100mer resin to 208bp or 1900bp DNA restriction fragments on the other hand was not affected by random base immobilisation. The results are illustrated in table 15.

The interference of random base immobilisation with hybridisation became most apparent, when 20mer target was used in reactions with the three oligonucleotide resins. Unexpected variation was found between the optimum hybridisation temperatures using the different resins.

Noyes and Stark found the relative binding efficiencies of nucleotide homopolymers to DBM activated resins to be : poly G > poly T > poly C = T (1975). Guanine residues were thought to bind twice as efficiently as thymine residues. Negligible binding was thought to occur with thymine and cytosine residues. The variation of optimum hybridisation temperatures seen in the results, could be explained if the guanine content of the immobilised oligonucleotides were taken into account. The immobilised 50mer had the highest guanine content and therefore probably the most bases involved in attachment to the solid support. This would explain the decreased optimum hybridisation temperature seen upon hybridisation with the 20mer target and also with the longer target sequences.

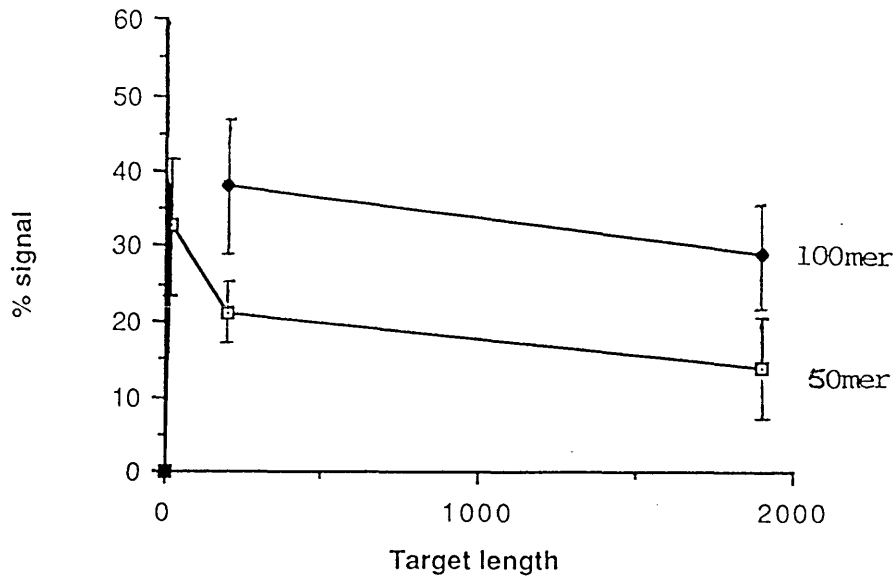
The one-step hybridisation results established that longer oligonucleotides would have to be used as capture probes for future sandwich hybridisation assays. The principal reason for this was that a sandwich assay would have to be carried out at 50°C, the optimum hybridisation temperature of the 20mer detector probe. Furthermore the assay would have to be stable at temperatures above 62°C, the temperature at which stringent washes would be conducted, for

differential hybridisation.

Other experiments suggested that longer oligonucleotide sequences would also improve target hybridisation efficiencies. The results are shown in table 16. The 100mer resin was more effective at capturing restriction fragments through hybridisation than the 50mer resin. Moreover the percentage hybridisations were also comparable to those obtained for immobilised calf thymus DNA, hybridising to complementary sequences. Non-specific hybridisations, with non-complementary target DNA (20mer), varied slightly between the 50mer and 100mer oligonucleotide resins. This variation was however thought to be attributed to the different oligonucleotide: resin coupling efficiencies. Both oligonucleotides were attached at different concentrations to the resin during immobilisation and therefore presented different amounts of surface area for non-specific binding.

Oligonucleotide resins with low concentrations of immobilised DNA were found not to affect target DNA hybridisation efficiencies. The increase in resin however did affect the amount of non-specific attachments. For future sandwich assays it was therefore important that only resins containing high concentrations of oligonucleotide were used.

The kinetics of hybridisation were examined through time course experiments, depicted in figure 46. Hybridisations were performed using 500fmol 100mer oligonucleotide resin and with 10 fmol free target DNA, at 65°C. The target lengths of 208 bp and 1900bp were 32 P kinase end-labelled. Non-complementary target DNA used for the controls was 1600bp in length. The results suggested that both complementary target lengths had reached maximum hybridisation within 30 minutes. The $t_{1/2}$ values for both hybridisations were measured to be less than 1 minute. Reduced flexibility and availability of the oligonucleotide capture probe upon diazotization probably affects the



Immobilised oligonucleotide "capture probe"	Target DNA	% Signal	% Background	fmol Target Hybridised
50mer (s)	20bp	33 ± 9	8	2.5
	200bp	21 ± 4	6	1.5
	1900bp	14 ± 6	4	1.0
	Control	8 ± 4		
.....				
100mer (as)	20bp	38 ± 9	3	3.5
	200bp	29 ± 7	3	2.2
	1900bp			
	Control	9 ± 4		
.....				
Calf Thymus DNA 300bp	300bp	43 ± 3	4	3.9
	Control	4 ± 4		

TABLE 16 ONE-STEP HYBRIDISATIONS USING TARGET DNA'S OF DIFFERENT FRAGMENT LENGTHS AND OLIGONUCLEOTIDE CAPTURE PROBES IMMOBILISED BY DIAZOTIZATION

Each hybridisation was set up with 500fmol immobilised oligonucleotide "capture probe" and 10fmol target DNA in a hybridisation volume of 50ul. The results are the mean results of a minimum of 2 experiments. The $\pm$ values refer to the maximum and minimum percent deviation around the established mean, recorded for each hybridisation reaction.

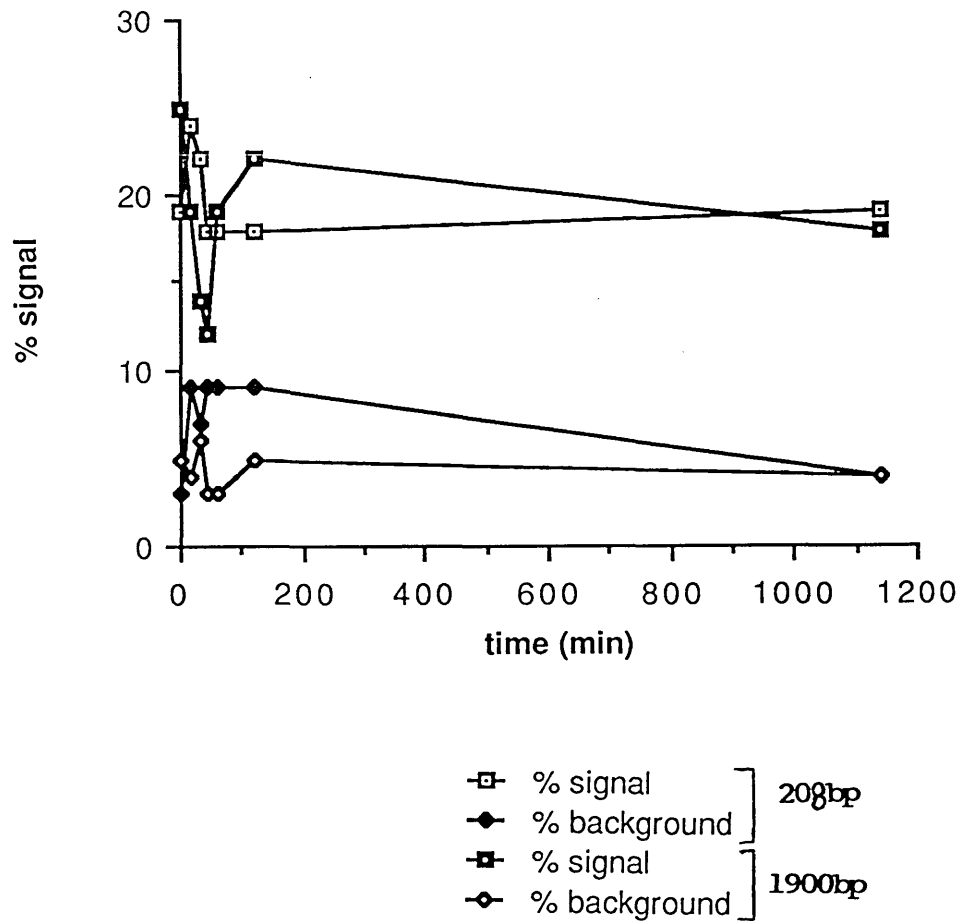


FIGURE 46 ONE-STEP HYBRIDISATION TIME COURSE USING THE CAPTURE PROBE IMMOBILISED BY DIAZOTIZATION AND COMPLEMENTARY TARGET DNA

The hybridisation reactions were performed with 500fmol 100mer beta-globin specific capture probe and 10fmol of complementary target sequences. The target sequences were restriction fragments of the recombinant plasmid Hbf-5, with lengths of 208 bp and 1900 bp. Hybridisations at time zero were measured by combining the reaction constituents on ice and removing these immediately through normal washing procedures (as described in the methods section). For both target lengths the $t_{1/2}$ values appear to be less than 1 minute.

Immobilised DNA (capture probe)	% One-step hybridisation			Immobilised DNA availability to S1 digestion (%)
	2nd wash	60°C wash	lost during hybridisation manipulations (%)	
Calf Thymus DNA (300bp)	46	39	16 $\pm$ 6	--
Control resin	18	8	--	--
Control target DNA (20mer)	10	3	--	--
.....				
100mer beta-globin	39	29	17 $\pm$ 10	69 $\pm$ 11
Control resin	21	6	--	--
Control target DNA (20mer)	34	4	--	--

TABLE 17 THE DNA LOSSES ASSOCIATED WITH ONE-STEP HYBRIDISATIONS, AND THE AVAILABILITY TO HYBRIDISATION USING CAPTURE PROBES IMMOBILISED VIA DIAZOTIZATION

Hybridisations were carried out using a fifty fold excess of the immobilised capture DNA (250 fmol) over the target DNA concentration (5 fmol). The percentage values are derived from the mean results of at least two sets of (duplicate) experiments. The resin used for capture probe immobilisation was Sephacryl S-1000. The target DNA's used were the 208 bp fragment coding for the sequence around the sickle mutation site of the beta-globin gene, and calf thymus DNA, 300 nucleotides in length. The immobilised DNA lost during hybridisation manipulations could either be the result of sheering effects or leakage of the covalently bound oligonucleotide from the support. The % DNA loss seen upon S1 digestion is a reflection of the capture probe sequence available to hybridisation once immobilised via diazotization. The +/- values relate to the maximum and minimum percentage losses recorded.

hybridisation kinetics. This could not, however, be confirmed with the results obtained (see figure 46).

Hybrid complexes formed with oligonucleotides immobilised by diazotization appeared to be stable complexes not easily destroyed by vigorous washing procedures. Stringent washes after hybridisation were associated with only a small loss in specific target DNA (table 17).

3.5.4. Summary

1. Short targets DNAs hybridised more readily and with greater stability to oligonucleotides than longer sequences.
2. Increasing the concentration of capture probe to 50-100 times in excess of the target concentration increased the efficiency of hybridisation.
3. One-step hybridisations were essentially completed in 30 minutes.
4. The surface area of the resins appeared to be the main cause for background binding of free target DNA.
5. The sensitivity of target detection was limited to 1 fmol. This was primarily due to the limited specific activity of the end-labelled target DNA.
6. Oligonucleotides immobilised enzymatically hybridised to complementary target oligonucleotides more efficiently than capture probes linked using carbodiimide. This difference was probably due to the presence of a spacer-arm in enzymatically immobilised DNA.

7. DNA immobilised by diazotization hybridised with greater efficiency and stability than DNA immobilised by other methods.
8. Random base immobilisation of oligonucleotides interferes with the optimum hybridisation temperatures.

3.6 Sandwich hybridisations with cloned target DNA

The one-step hybridisations demonstrated that oligonucleotides immobilised by diazotization were likely to present the most efficient capture probes. Consequently diazotized resins were used, initially to determine the properties of sandwich hybridisation.

The preliminary experiments were set up using the cloned beta-globin gene as the target DNA, and the pair of ^{32} P Kinase end-labelled globin specific oligonucleotides as detector probes, described earlier. A representation of the sandwich assay is depicted in figure 47. The conditions required for differential hybridisation of the detector probe were established, as well as the time required for maximum hybridisation. The possible benefits of using the oligonucleotide probe, containing 2-aminoadenine, in place of the ordinary probe were assessed. A comparison was also made to a sandwich hybridisation assay using enzymically immobilised oligonucleotide capture probe.

Sandwich hybridisations were assumed to be 100% efficient when all of the theoretically available target DNA was taken up in the hybridisation reactions. These estimates did not take into account the one-step hybridisation efficiencies of the target DNA.

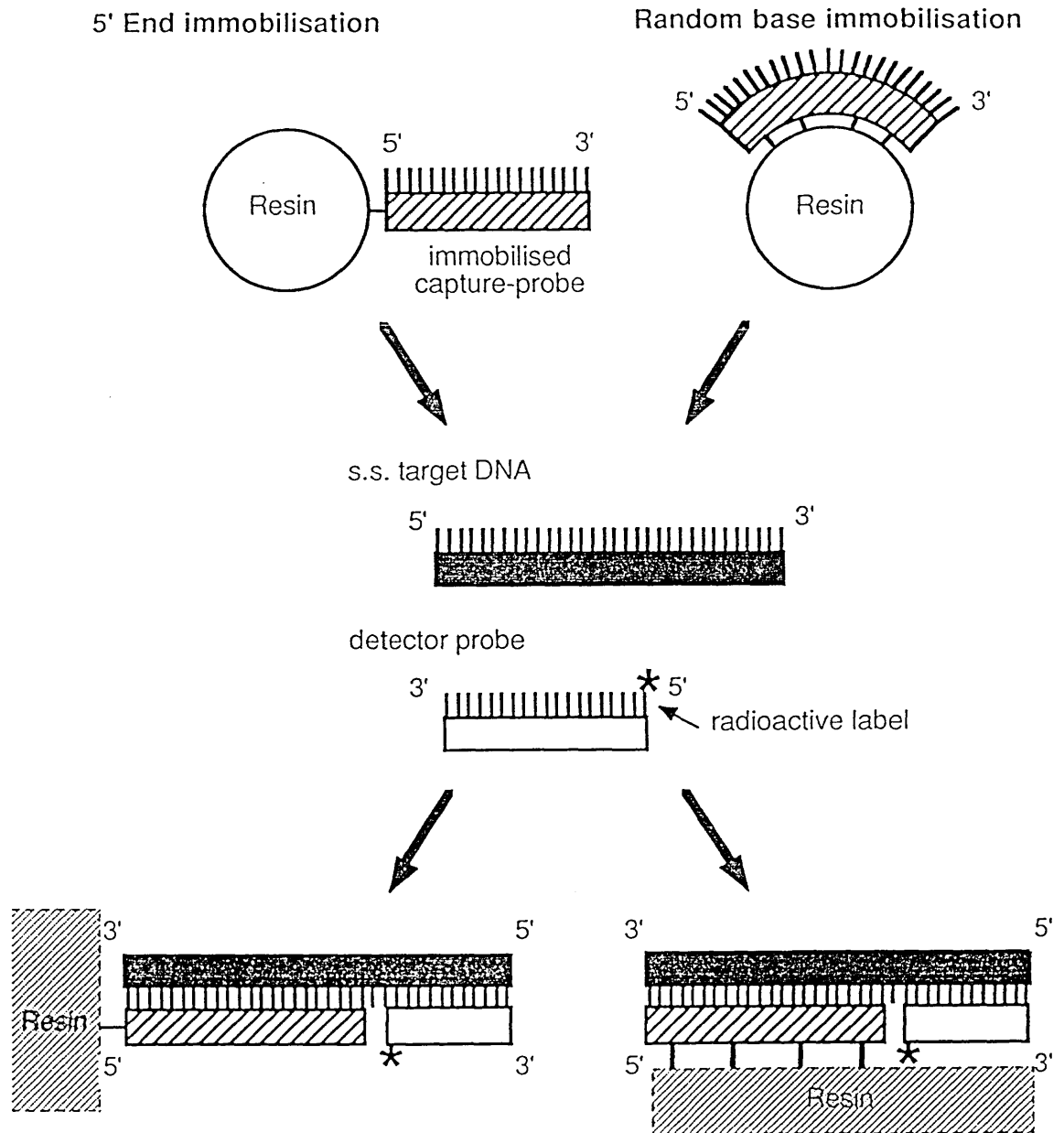


FIGURE 47 SANDWICH HYBRIDISATION WITH AN IMMOBILISED OLIGONUCLEOTIDE CAPTURE PROBE

3.6.1 Properties of a sandwich hybridisation assay

The conditions used for sandwich hybridisation were determined by the optimum conditions of the detector probe. The resin complexes were subjected to stringent washing manipulations upon hybridisation to remove unbound detector probe, and to remove the mismatched hybrid duplexes. Generally three washes followed by a stringent wash at 60°C were sufficient for detector probe differentiation of target genotypes.

The initial sandwich hybridisation experiments used 250 fmol 100mer oligonucleotide (anti-sense strand) resin, 5 fmol target DNA and 250 fmol 32 P Kinase end-labelled detector probe (antisense-strand). The results presented in table 18. are the mean percentage hybridisations established with beta-globin target lengths of both 208bp and 1900bp. The $\pm$ values are the maximum and minimum deviations about the mean % hybridisations recorded. The hybridisation efficiencies were obtained by comparing final Cerenkov counts of the hybridised detector probe, with the initial counts. Also taken into consideration was the factor that only 2% of these could bind to the target DNA. Figure 48. is a diagrammatic representation of this sandwich assay using the 208bp recombinant target DNA.

The non-specific attachments were established by three controls; 1) using resins without immobilised capture probe; 2) omitting the target DNA from the reaction and 3) using a non-complementary detector probe (the alpha-1-antitrypsin 20mer). The first control was a direct reflection of the total background binding. The second revealed the background binding that could be attributed to the non-specific binding of the detector probe. It is interesting to note that in the absence of target DNA a greater concentration of background was generated, suggesting that

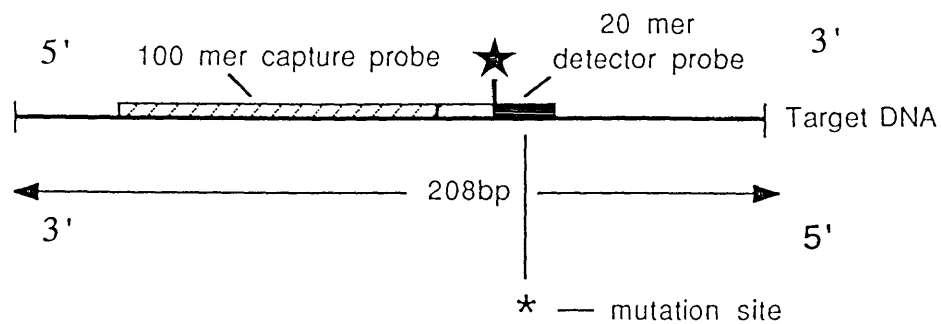


FIGURE 48 A DIAGRAMATIC REPRESENTATION OF THE SANDWICH ASSAY USING 208bp RECOMBINANT TARGET DNA AND TWO OLIGONUCLEOTIDE PROBES.

One of the two oligonucleotide probes was immobilised to the solid beaded support and acted as the target capture probe. The second oligonucleotide probe was ^{32}P kinase end-labelled (*) and served as the detector probe. Both oligonucleotides were non-overlapping sequences, with the capture probe upstream in sequence (antisense strand) to the detector probe and the mutation site being analysed.

oligonucleotides are the prime contributors to non-specific binding. The third control helped determine how much of the detector probe was specifically hybridised to the target DNA.

Pre-hybridisation was essential in that it reduced the background binding events, (data not shown). Both long DNA fragments and short oligonucleotides (10mers) were found to be the most effective blocking agents. Prolonged Pre-hybridisation was found to be unnecessary as non-specific binding appeared to occur almost immediately upon addition of the labelled detector probe to the resin. The addition of 5x Denhardt's solution to the hybridisation mix, to act as a blocking agent, had little effect on non-specific background binding.

A two-fold difference in the duplex formation of matched with mismatched beta-globin specific detector probes was obtained, when the 208bp target was used (table 18). At first sight this differential hybridisation was not as clearly defined when the 1900bp target fragment was used. This however can be seen to be the result of an increase in non-specific binding. Subtracting the total background percentage binding from the hybridisation results, reveals a similar differentiation of the target sequences. The positive identification of matched duplexes was associated with a signal at least two and a half times the maximum background signal.

Initially, the ratio of 50:1:50 was used for the concentrations of immobilised capture probe, to target DNA, and to detector probe. Previous one-step hybridisation results with labelled target DNAs suggested that this ratio might be suited for efficient sandwich hybridisation. To establish whether this was true, experiments were set up with different amounts of either free probe or target DNA.

Using 250 fmol 100mer oligonucleotide resin and 5 fmol of the 208bp target DNA, hybridisations with different detector probe

Target length	Target DNA	Detector DNA	% Signal
208bp	globin	globin	33 + 10
	globin	sickle	14 + 7
	globin	control 20mer	9 + 6
	sickle	sickle	27 + 5
	sickle	globin	13 + 6
	sickle	control 20mer	7 + 5
	none	globin	11 + 4
	Control resin	globin	3 + 2
			
1900bp	globin	globin	28 + 8
	globin	sickle	13 + 6
	globin	control 20mer	16 + 5
	sickle	sickle	31 + 4
	sickle	globin	16 + 3
	sickle	control 20mer	11 + 4
	none	globin	11 + 4
	Control resin	globin	6 + 3

TABLE 18 SANDWICH-HYBRIDISATIONS USING 100MER CAPTURE PROBES IMMOBILISED BY DIAZOTIZATION, SINGLE STRANDED TARGET DNA AND 20MER DETECTOR PROBES COMPLEMENTARY TO THE SICKLE MUTATION SITE OF THE BETA-GLOBIN GENE

The hybridisations were performed overnight at 50° C, in a total reaction volume of 50ul. Stringent washing manipulations enabled differential hybridisation of the 32P kinase end-labelled detector probe to the recombinant target DNA (208bp and 1900bp). The results show a clear differentiation between the normal and the sickle target genotypes, the former generating a hybridisation signal two fold greater than the latter.

concentrations were performed. To determine the extent of non-specific hybridisations non-complementary detector probe (20mer) was used in control assays. The results depicted in figure 49 illustrate that increased detector probe concentrations did not significantly alter the efficiency of hybrid formation. This increase did however affect the signal:background ratio, which decreased with increasing detector probe concentrations.

Experiments with 250 fmol 100mer oligonucleotide resin and detector probe were set up to ascertain the effect of changing target concentrations. Non-specific hybridisations were again established using a non-complementary detector probe (20mer) in control assays. With an increase in target DNA a decrease in hybridisation was observed, along with a respective increase in background attachments (figure 50). This suggested that the optimum target concentration was in fact similar to the one recorded for one-step hybridisations. The ideal hybridisation system thus appeared to be one containing an excess of immobilised capture probe (at least 50 fold), bound to minimal amounts of resin, and an excess of detector probe (less than 10 fold) over the target DNA.

Hybridisation efficiencies were not improved with 10% dextran sulphate, which acts on one-step hybridisations favourably by increasing the viscosity and therefore the apparent concentration of the detector probe (Gingeras 1987). Similarly neither the efficiencies nor the background were improved by incubating the target DNA and the detector probe sequentially in a two-step assay.

Having determined the optimum conditions for hybrid formation a time course was performed to ascertain the minimum time required for sandwich hybridisation. Again a 50 fold excess of the 100mer oligonucleotide resin as well as the detector probe was used together with 10 fmol 200bp target DNA. The results shown in figure 51 indicate

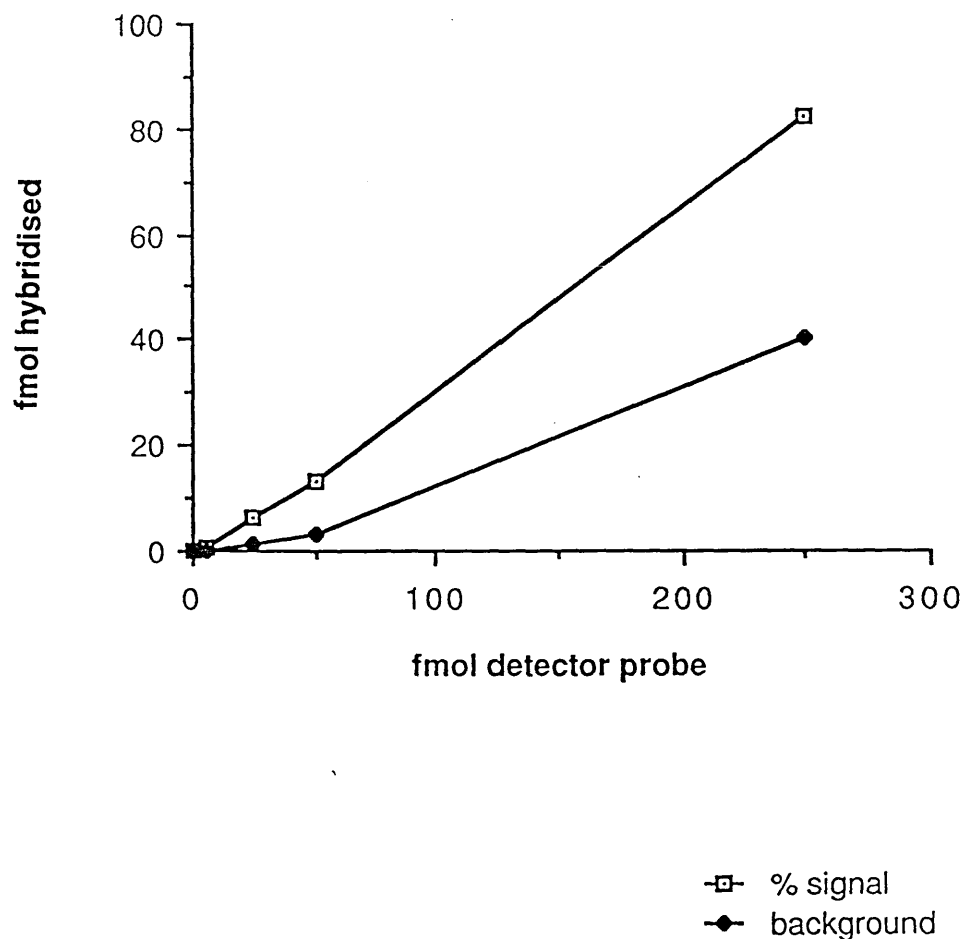


FIGURE 49 THE EFFECT OF INCREASING THE DETECTOR PROBE CONCENTRATION IN A SANDWICH HYBRIDISATION ASSAY USING 100MER CAPTURE PROBES IMMOBILISED BY DIAZOTIZATION AND 208 BP TARGET DNA

Hybridisations were performed with 250 fmol, 50 fmol, 25 fmol and 5 fmol of the beta-globin specific 20mer detector probe. The reactions were carried out using 250 fmol each of the capture probe immobilised by diazotization and 5 fmol of the 208bp globin restriction fragment derived from Hbf-5, in a volume of 100ul. The hybridisations were incubated overnight at 50°C. The results show that the greater the excess of the detector probe over the target DNA the greater the hybridisation efficiencies. This increase is however coupled with an increase in background, non-specific hybridisations.

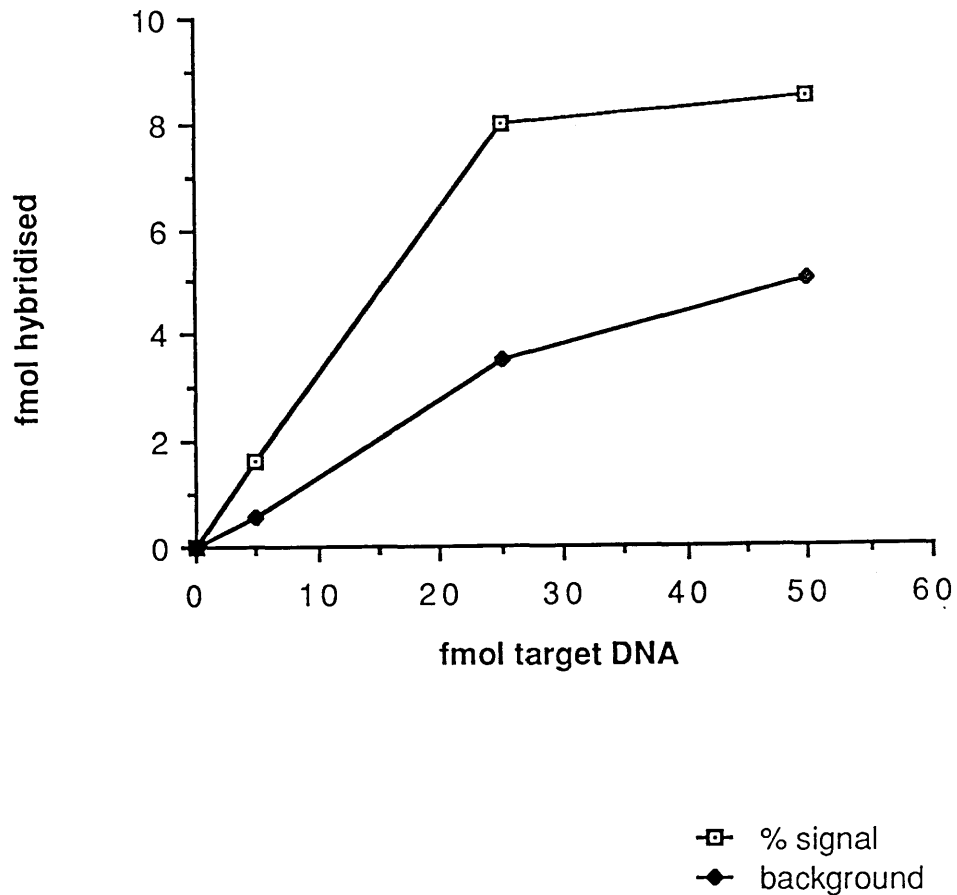
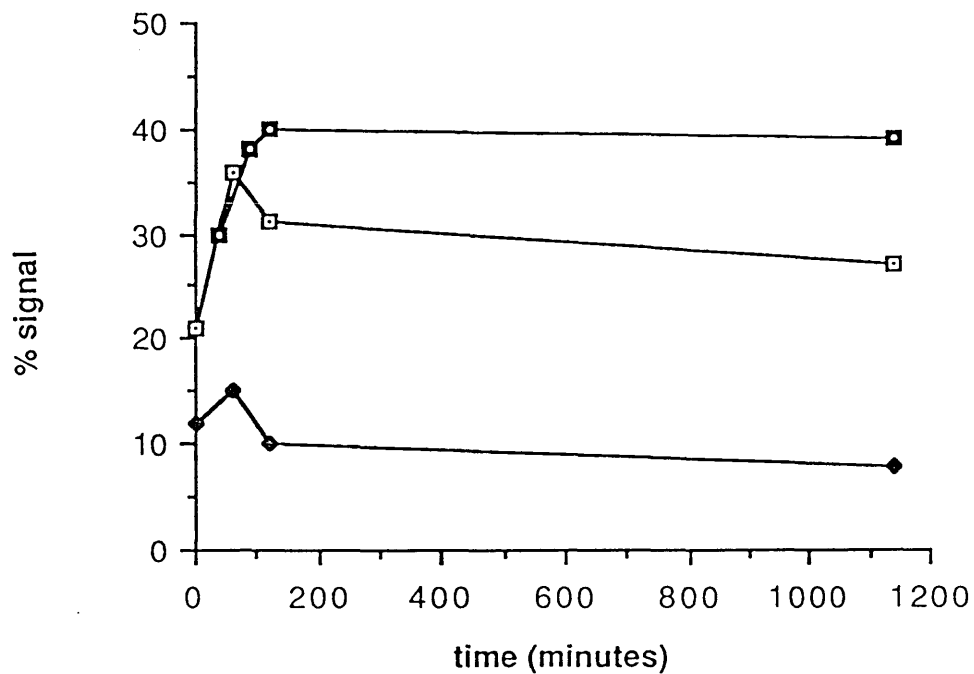


FIGURE 50 THE EFFECT OF INCREASING TARGET DNA CONCENTRATIONS IN A SANDWICH-HYBRIDISATION ASSAY, USING CONSTANT AMOUNTS OF BOTH DETECTOR AND CAPTURE PROBES

Hybridisations were performed with 50 fmol, 25 fmol and 5 fmol of the 208 bp target fragment derived from the Hbf-5 recombinant plasmid. The reactions were carried out using 250 fmol each of the capture probe immobilised by diazotization and the ^{32} P kinase end-labelled detector probe, in a volume of 100 μ l. The hybridisations were incubated overnight at 50°C. The results show that increased target concentrations are coupled with an increase in the non-specific hybridisations. They also show that a greater proportion of the input concentration of target DNA is immobilised when capture and detector probe are present in excess.



detector_probes

- globin
- ◆ sickle
- analogue

FIGURE 51 A COMPARISON OF THREE SANDWICH-HYBRIDISATION TIME COURSES USING THREE DIFFERENT DETECTOR PROBES THE 100MER CAPTURE PROBE IMMOBILISED BY DIAZOTIZATION, AND THE NORMAL BETA-GLOBIN 208BP RECOMBINANT TARGET DNA

The hybridisations were carried out at 50°C and with the following capture probe, target DNA and detector probe concentrations: 500 fmol:10 fmol:500 fmol respectively. From the results the optimum differential hybridisation time appears to be between 1 and 2 hours. A slight decrease in the hybridisation efficiencies is seen upon overnight incubation.

that maximum hybridisation was essentially reached in one hour. It was also from this point of maximum hybridisations that a slight drop in signal was often observed, after an overnight incubation. This behaviour may have been caused by strand displacement of the target DNA, induced by the prolonged incubation time. The background values were obtained from non-specific hybridisation controls, performed with non-complementary 20mer oligonucleotide.

3.6.2 The benefits of using detector probes containing the analogue 2-aminoadenine

Dot blot hybridisations with immobilised target DNAs had revealed an increase in duplex stability when the analogue containing oligonucleotide probe was used. This expressed itself through an increase in melting temperature of the hybridised analogue containing probe when compared to the normal oligonucleotide probe. It was thought that this property would improve differential hybridisation in a sandwich assay.

The 20mer beta-globin detector probe containing 2-aminoadenine was used in sandwich assays and its properties were compared with those of an ordinary 20mer detector probe. Sandwich hybridisations performed at a temperature of 50°C found the efficiencies of hybridisation to complementary target DNA to be similar to those of the normal 20mer probe. Stringent washing manipulations however found matched hybrids to be more stable with analogue containing detector probes as part of the duplex structure.

This stability was more apparent when the hybridisations were carried out at 55°C. At this temperature a greater differential hybridisation between matched and mismatched target sequence could be achieved. The difference in hybridisation was also greater than that

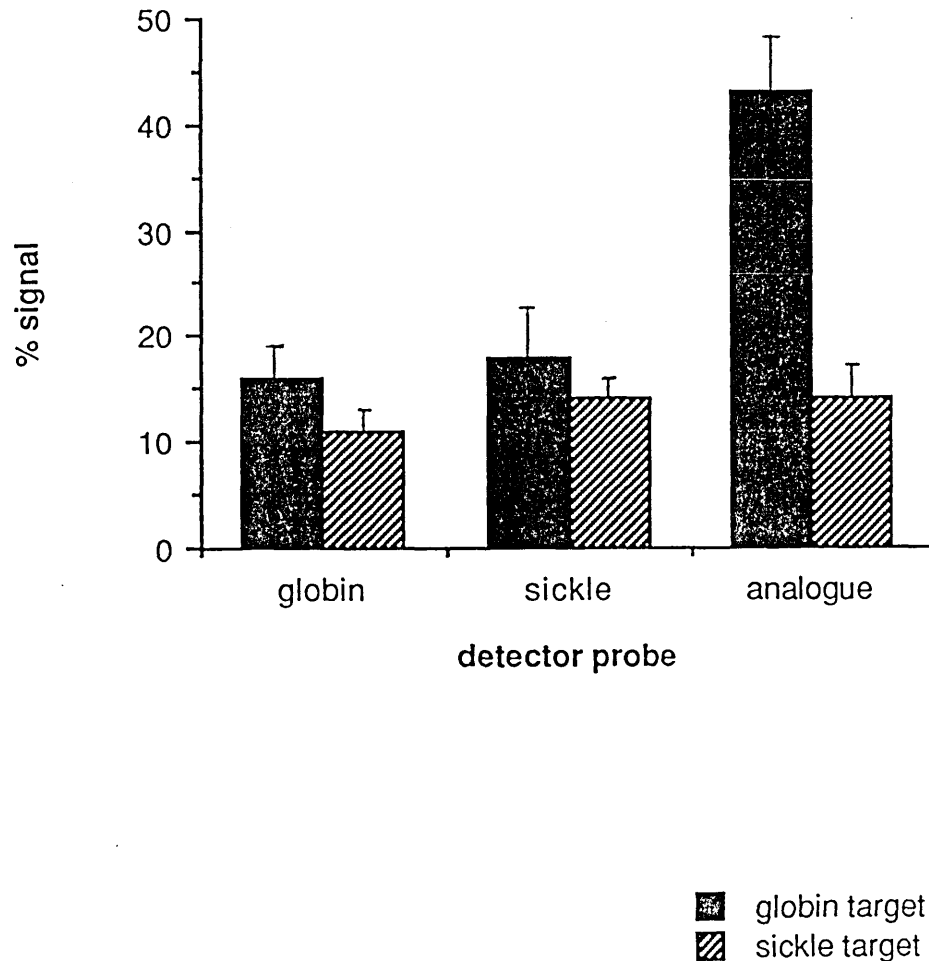


FIGURE 52 A COMPARISON BETWEEN THE HYBRIDISATION PROPERTIES OF AN ANALOGUE CONTAINING PROBE AND A NORMAL DETECTOR PROBE IN A SANDWICH-HYBRIDISATION ASSAY

The assay was carried out using the 100mer capture probe (200 fmol), immobilised by diazotization, the 208 bp (4 fmol) recombinant target DNA and a radioactively kinase end-labelled 20mer detector oligonucleotide (200 fmol). Hybridisations were carried out at a stringent hybridisation temperature of 55°C. No differential hybridisation was achieved using the normal, wild type and sickle 20mers at this temperature. The results show that the analogue containing detector probe could be used to generate a greater differentiation in the hybridisation to normal and single base mutated target sequence, than probes without the analogue. The error bars show the maximum percentage deviation from the mean recorded for each of these hybridisation reactions.

which could be obtained at lower temperatures with normal detector probes. A four fold difference in positive signal over negative signal was found, as is illustrated in figure 52. The non-specific background (minus appropriate target or resin) and hybridisation controls (using a non-complementary 20mer probe) for this assay were found to result in 5-10% signal binding.

The time course results depicted in figure 51 show that the substitution of adenine for 2-aminoadenine did not alter the hybridisation kinetics of the detector probe in a sandwich assay. The reactions performed at 50°C also illustrate the insignificant difference in hybridisation efficiencies between the analogue containing probe and normal probe, at this temperature, as described earlier.

3.6.3 Sandwich hybridisation with enzymically immobilised oligonucleotide resins

Sandwich hybridisation efficiencies of capture probe immobilised enzymically were compared with the results obtained for capture probe immobilised by diazotization. Hybridisations were performed using 50mer (sense-strand) enzymically end-immobilised capture probe and 20mer PCO 4 detector probe (sense-strand). The PCO4 sequence used coded for the antisense strand of the beta-globin gene down stream in sequence (36bp from the 3' end) of the sickle cell mutation site.

Detector probe was used 100 times in excess over the target concentration and the capture probe, in turn was used 50 times in excess over the target DNA. One-step hybridisation results with 1900bp beta-globin target DNA resulted in high one-step hybridisation efficiencies (37-44%). The results displayed in table 19, are also

One-step target hybridisation:

Target sequence	Target length	% hybridised	pmol bound
Normal	1900bp	37 $\pm$ 4	0.74
Sickle	1900bp	44 $\pm$ 7	0.88
Control	1600bp	8 $\pm$ 5	0.16

Two-step detector probe hybridisation:

		% detector probe hybridised	pmol detector probe bound
Normal	1900bp	14 $\pm$ 5	0.28
Sickle	1900bp	15 $\pm$ 2	0.3
Control	1600bp	9 $\pm$ 4	0.18

TABLE 19 SANDWICH-HYBRIDISATION USING THE 50MER BETA-GLOBIN CAPTURE PROBE,IMMOBILISED THROUGH ENZYMATIC LINKAGE AND THE PCO 4,20MER DETECTOR PROBE

The detector probe (PCO4) was used 100 times in excess (200 fmol) over the target DNA concentration (2 fmol).The capture probe (50mer) was present in 50 fold excess (100 fmol) over the target DNA concentration.Hybridisations were carried out over night at 50°C,however no differential hybridisation was expected between the beta-globin normal and the sickle target DNA's,as the PCO 4 probe was complementary to a sequence up-stream from the sickle mutation site.The results display sandwich-hybridisation yields which are considerably less than those seen when the capture probe is immobilised by diazotization.

similar sandwich hybridisation efficiencies, to those recorded for hybridisations with the same target length and 50mer capture probes immobilised by diazotization. They however did not compare with the efficiency of target capture which could be achieved with the 100mer probe immobilised by diazotization (30-40%).

Although the results obtained in these reactions with enzymically immobilised capture probe were good, the overall stability of the sandwich hybrid still appeared to be less, than the hybrid complex which could be achieved with oligonucleotide probes immobilised by diazotization. Repeated stringent washing manipulations readily destabilised the hybrid complex. More efficient sandwich hybridisations with enzymically immobilised capture probe might have been obtained with longer capture probe sequences.

The oligo (dT) cellulose resin with a 12-18bp oligo (dT) spacerarm, used in this experiment, was found more suited to sandwich hybridisation than the resin with a 8-12bp spacerarm, used in other experiments. This might support the view that a spacer-arm adds to the stability of the hybridisation duplex, but may also be a reflection of increased hybridisation efficiency with longer capture probe sequences.

3.6.4 Summary

1. The hybridisation efficiencies of matched hybrid complexes within a sandwich assay, using capture probes immobilised by diazotization and 208bp target DNA, were between 30-40%. Non-specific hybridisations were around 10%.
2. Mismatched hybrid duplexes between target DNA and detector probe could be removed through stringent washing procedures.

3. Maximum hybridisation could essentially be achieved in 1h.
4. Optimum hybridisation conditions required the capture probe concentration to be at least fifty times more than that of the target DNA.
5. The amount of background binding to the resin was directly proportional to the concentration of the detector probe present in the hybridisation reaction. Increased detector probe concentrations however resulted in increased hybridisation efficiencies.
6. By incorporating the analogue 2-aminoadenine into the detector probe a greater hybrid stability, particularly at elevated temperatures was achieved. A greater differential hybridisation of detector probe to matched and mismatched sequences could be obtained.
7. Enzymically immobilised capture probe appeared to form less stable sandwich hybrid complexes than probe immobilised by diazotization.

3.7. Polymerase chain reaction

One of the limitations encountered in the development of the oligonucleotide based sandwich assay was in the detection of small concentrations of target DNA (>1 fmol). In order for the oligonucleotide detector probe to detect single copy genes, the use of labelling strategies, incorporating ^{32} P to higher specific activities would have been required. The development of the polymerase chain reaction (PCR) by Saiki et al. (1986) provided an alternative solution to this problem. Specific PCR amplification of the target DNA from

attomolar to femtomolar concentrations, using a polymerase enzyme and sequence specific primers, would eliminate the need for probes radioactively labelled to high specific activities.

Saiki et al. had developed the method of PCR amplification using the beta-globin gene, around the site of the sickle cell disease mutation. The 20mer primers used, PCO3 and PCO4, lead to the generation of a 110bp fragment. Two polymerase enzymes could be used for this amplification of target DNA as follows: 1) the large Klenow fragment of Escherichia coli DNA polymerase I, and 2) Taq DNA polymerase I, a heat stable enzyme (Chien et al, 1976).

Using both polymerase enzymes and the same amplification primers illustrated in figure 53, the recombinant beta-globin gene and human beta-globin gene from patient DNA samples were amplified.

Klenow enzyme was used in a manual amplification technique, and automated amplification was performed with the Taq DNA polymerase I enzyme. Generally manual amplification was not as reliable and did not result in yields as high as could be obtained through automated amplification. After 30 cycles of amplification (one cycle extending from one heat denaturation to another) a theoretical yield of 1×10^9 fold amplification, equivalent to 39 ug of 110mer specific sequence was expected. Manual amplification yields however, were between 0.1 ug-1 ug in comparison to the automated yields of between 1-3ug.

A possible explanation for this could have been that primer sequences eventually became limiting to the reaction. This may have occurred more rapidly if a significant amount of non-specific primer attachments had occurred. With the low temperatures (37°C) required for optimum activity of the Klenow enzyme, this could have been expected. The decreased yields found using the Klenow enzyme may also have been the result of insufficient target denaturation at several stages throughout PCR amplification. The reaction temperature was not as

1. PCO 3: ACA CAA CTG TGT TCA CTA GC
(upstream from the sickle mutation site)
2. PCO 4: CAA CTT CAT CCA CGT TCA CC
(downstream from the sickle mutation site)

(Saiki et al.,1985).

FIGURE 53 THE PRIMER SEQUENCES USED FOR PCR AMPLIFICATION OF THE β GLOBIN GENE AROUND THE SITE OF THE SICKLE MUTATION.

The two 20mer primers used for amplification of the beta-globin gene around the sickle cell mutation site. PCO 3 and PCO 4 are derived from both the anti-sense and the sense DNA sequence strands respectively. The primers generate a target fragment size of 110 nucleotides upon PCR amplification.

easily regulated as in the automated technique, and may not have reached duplex melting temperatures on several occasions.

To verify that the PCR amplified (human genomic) DNA was beta-globin specific, direct gel hybridisations with complementary oligonucleotide were carried out (see figure 54). The double-stranded target DNA (human genomic) was also digested with Dde I restriction enzyme and hybridised to end-labelled probes. This reaction generated two double stranded fragments of approximately 55bp, which were seen to migrate to the same position as unrestricted single stranded 110bp target, during electrophoresis. The results are depicted in figure 55.

The buffer found to be most suited for PCR amplification is described in the materials and methods section. Buffers containing DMSO did not increase amplification yields. Primer and target concentrations on the other hand did affect the yields. Excess target DNA appeared to inhibit the reaction as can be seen in figure 56. An increase in target DNA (recombinant DNA), from 164 ng -1000 ng resulted in a noticeable decrease in amplification. Increased concentrations of primer appeared to increase the non-specific PCR amplification of other sequences (data not shown). By increasing the annealing temperature a considerable amount of these non-specific attachments could be prevented.

The minimum amount of target DNA (human genomic) which could be PCR amplified was determined through dilution experiments, reducing the initial target DNA input concentration. The DNA synthesised was run out on an agarose gel and was visualised through ethidium bromide staining. Six molecules of initial starting material, when PCR amplified with Taq DNA polymerase I, resulted in amplification that could easily be detected (data not shown). The estimated starting concentration of DNA was equivalent to 120 fmol, or 8.7 ng DNA.

To establish whether amplification could also be carried out

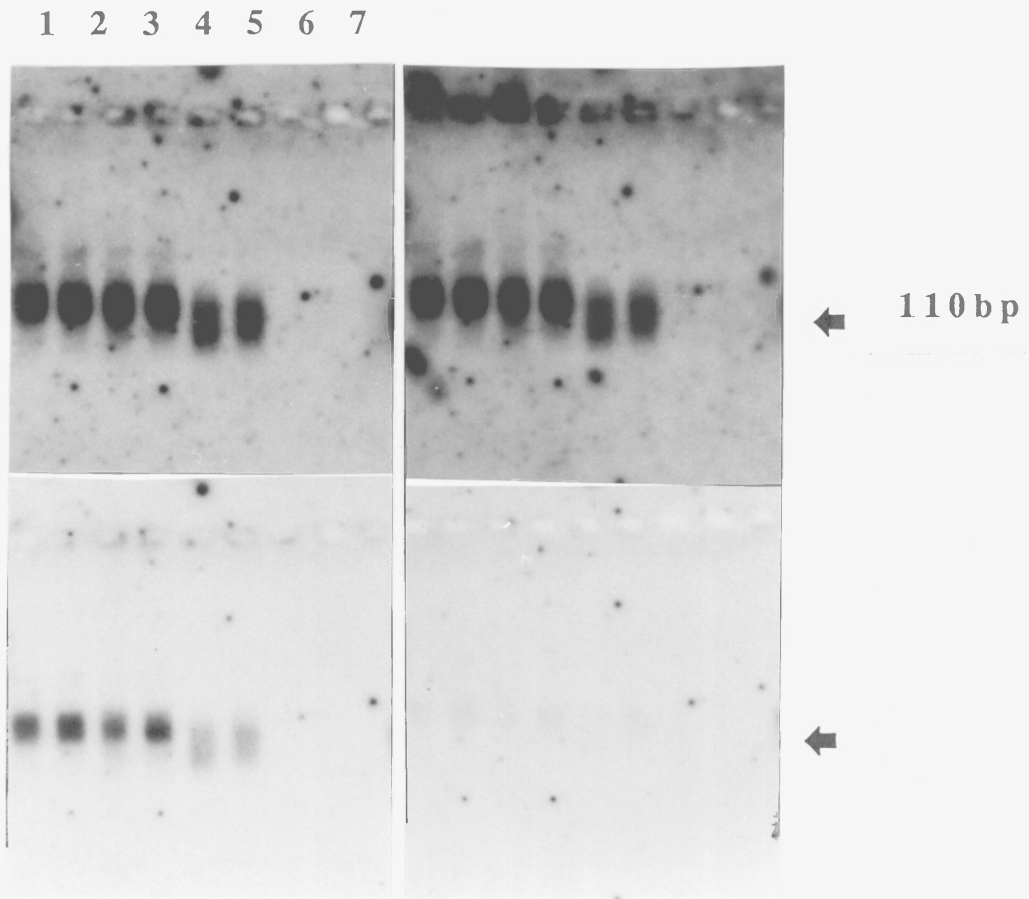


FIGURE 54 DIRECT GEL HYBRIDISATION OF A 20MER OLIGONUCLEOTIDE PROBE TO PCR AMPLIFIED β -GLOBIN TARGET DNA

The first two lanes (1,2) contain DNA subjected to 30 cycles, the next two (3,4) to 20 cycles and the last two (5,6) to 10 cycles of PCR amplification (manual) using the PCO 3 and PCO 4 primers, designed to amplify a 110bp region of the β -globin gene around the sickle mutation site. The starting concentration of target DNA in each sample was 5 μ g, the concentration of primers 100 pmol (0.65 mM), and the polymerase enzyme used was the large klenow fragment of DNA polymerase I. The 1% agarose gel was loaded with half of the entire reaction volume for each sample and was then subjected to electrophoresis, followed by denaturation and drying for hybridisation. The oligonucleotide detector probe used was 10 pmol of the 20mer β -globin specific sequence, which was ^{32} P kinase end-labelled. Stringent washes at 50 $^{\circ}$ C, 60 $^{\circ}$ C and 64 $^{\circ}$ C resulted in dissociation of the hybrid duplex, indicating that the PCR amplified sequence was β -globin specific and of the normal genotype. Lanes 7 and 8 contain control samples where primers and target DNA respectively were omitted from the PCR reaction.

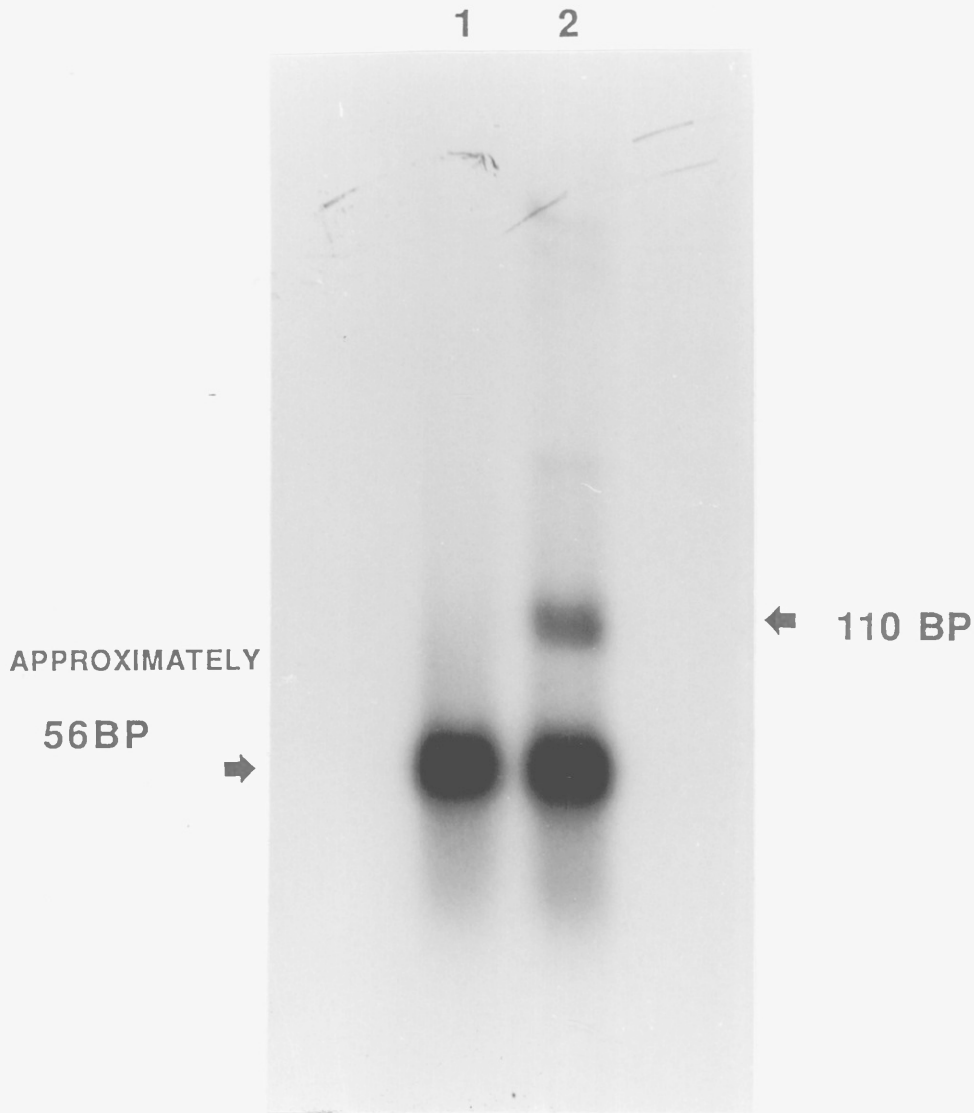


FIGURE 55 A *Dde I* RESTRICTION DIGEST OF THE 110 bp β -GLOBIN SPECIFIC FRAGMENT GENERATED THROUGH PCR AMPLIFICATION, HYBRIDISED TO A 20MER OLIGONUCLEOTIDE BY DIRECT GEL HYBRIDISATION

Lane 1 contains the *Dde I* digested fragment of 110 bp generated through PCR amplification. The two restriction fragments generated are 53 and 59 bp long. The results show the co-migration of the two fragments as one band in the 1% agarose gel used. Single-stranded forms of the target DNA will have also comigrated with these restriction fragments. Lane 2 contains the undigested 110 bp DNA having migrated both in the single and the double-stranded form during electrophoresis. High molecular weight β -globin specific strands are also present in this sample and are probably the result of secondary supercoiling. Of the 20mer globin specific oligonucleotide used 10 pmol was used as a probe for hybridisation and was ^{32} P kinase end-labelled.

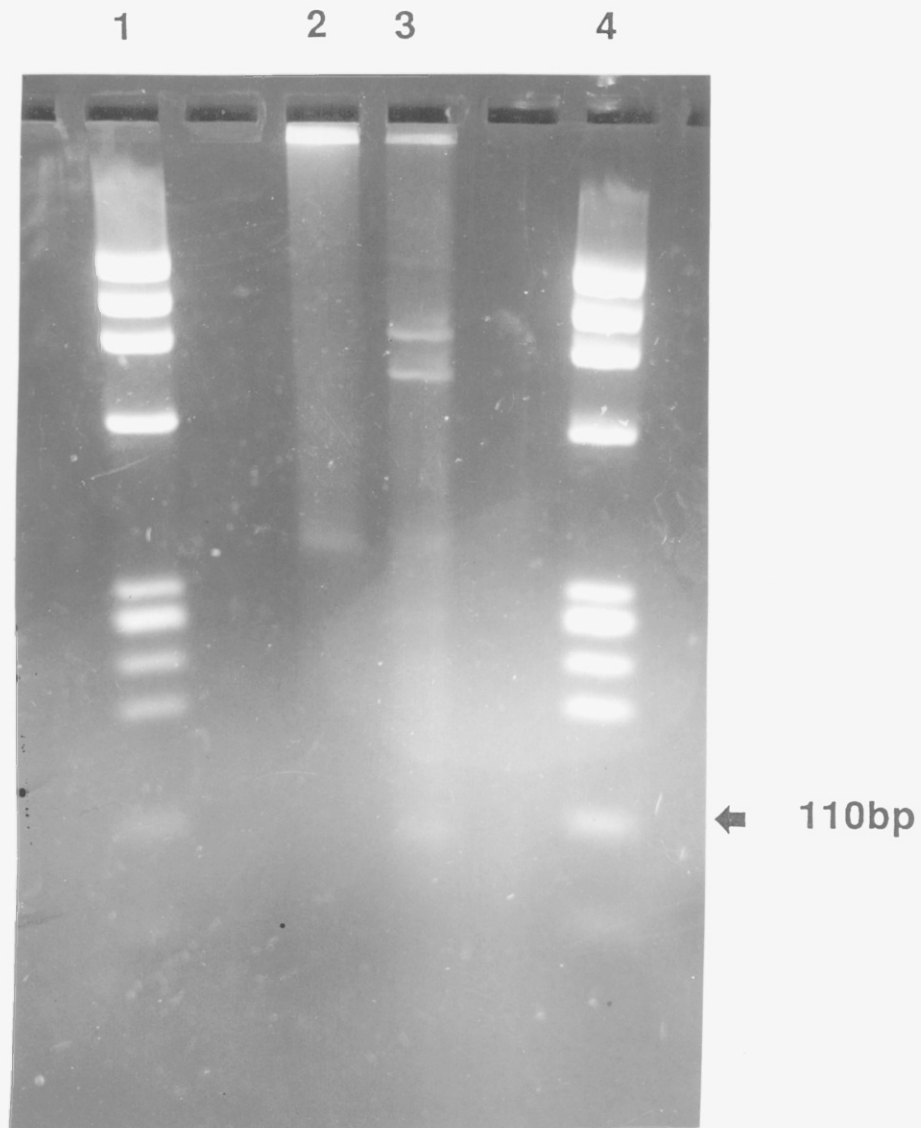


FIGURE 56 ETHIDIUM STAINED AGAROSE GEL OF PCR AMPLIFIED H β f5 AND H β S-1, BOTH AMPLIFIED USING DIFFERENT DNA STARTING CONCENTRATIONS.

Lanes 1 and 4 contain Hae III digested ϕ X174. Lanes 2 and 3 contain H β f5 and H β S-1 PCR amplified DNA respectively. Both DNA samples were amplified using PCO 3 and PCO 4 primers, generating a 110bp β -globin fragment. The initial DNA concentration of each sample was 1 μ g and 164 ng respectively, and both DNA samples were amplified in the closed circular form. The results show a preferential amplification of the sickle DNA sample, suggesting that smaller DNA concentrations favour amplification.

with less purified DNA samples two experiments were performed. In one experiment, the target DNA (human genomic) was extracted with proteinase K as described in the material and methods section. Assuming that an average of 120 white cells were present in each microlitre of crude human blood, 480, 960, 1920, 3840, and 7680 cells were exposed to this method of extraction and then PCR amplified with Tag DNA polymerase I. The polymerase enzyme was added after the initial heat denaturation step.

A second experiment used 25 and 30 ul human blood (3000 and 3600 white cell equivalent), diluted with an equal volume of Tris HCl buffer pH 7.0. The samples were heat denatured for 10 minutes and then exposed to amplification with Tag DNA polymerase I. The results of agarose gel electrophoresis suggested that no amplification had taken place. After hybridisation with a globin specific oligonucleotide some amplification could be detected.

As part of a blind study, samples of human genomic DNAs were amplified around the beta-globin gene using the Tag DNA polymerase I enzyme. The DNAs used were derived from three blood samples of human DNA, each coding for one of the three beta-globin genotypes. The genotypes of the DNA samples (0.5 ug per sample) were recorded but were not referred to during these experiments.

Similar concentrations of the 110bp fragment appeared in each track, when 5 ul of the unknown PCR amplified patient samples, separated by agarose gel electrophoresis, were stained with ethidium bromide, as can be seen in figure 57. Direct gel hybridisation of the samples, to complementary 20mer oligonucleotide probes, enabled differentiation of the three unknown genotypes (figure 58).

It is interesting to note that the high molecular weight, fragments visible upon ethidium bromide staining of the agarose gel, repeatedly appeared in PCR amplified patient samples of the same

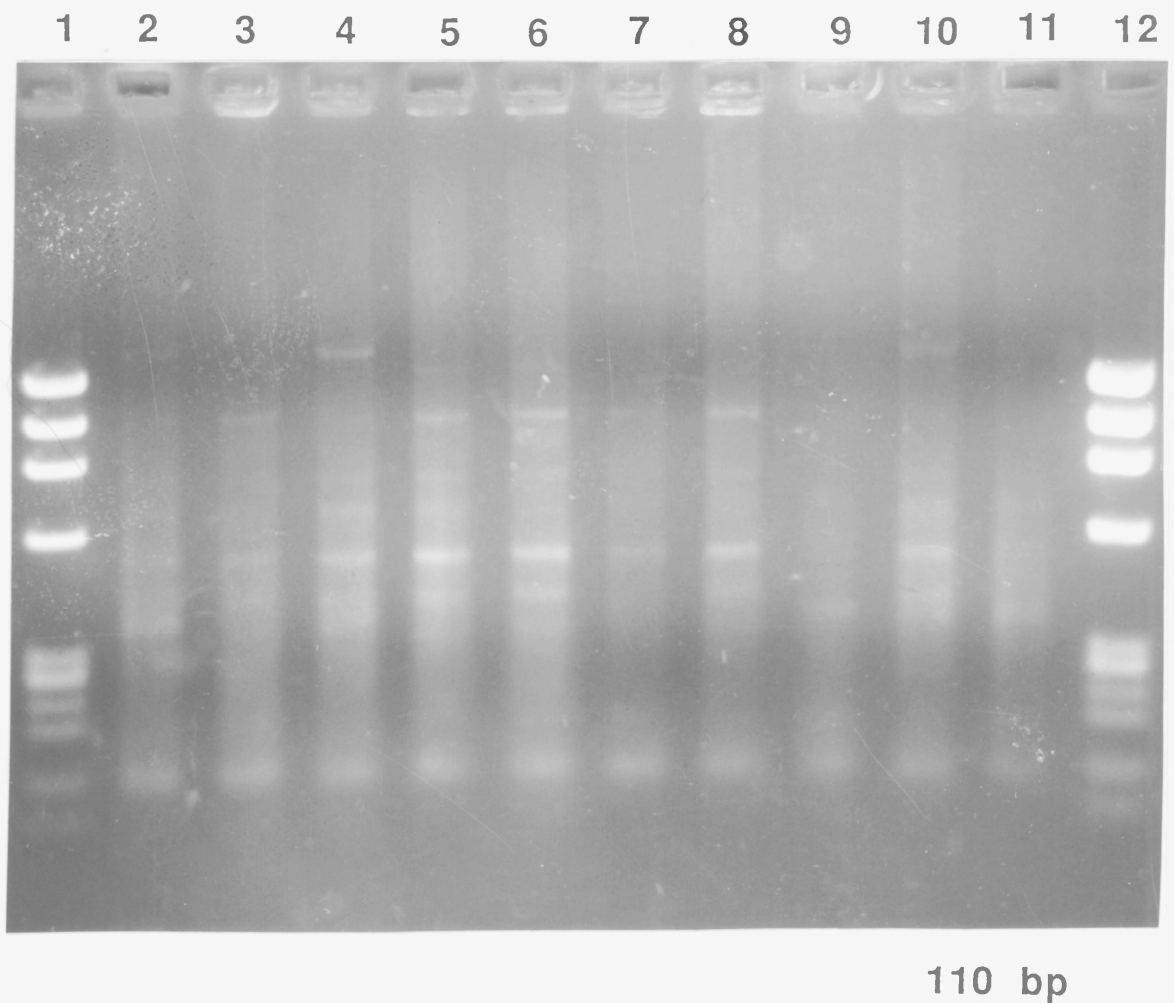


FIGURE 57 AN ETHIDIUM STAINED AGAROSE GEL SHOWING THE 110 bp β -GLOBIN SPECIFIC FRAGMENT OBTAINED THROUGH THE PCR AMPLIFICATION OF PATIENT DNA SAMPLES USING PCO 3 AND PCO 4 PRIMERS

Each sample was subjected to fifty cycles of PCR amplification (automated) using a DNA starting concentration of 0.5 ug. A 3% Nu Sieve agarose GTG /1% normal agarose gel was loaded with one tenth of the contents of each reaction tube and subjected to electrophoresis for a few hours. The gel was loaded as follows: 1 and 12 are ϕ X174 *Hae III* markers and lanes 2-10 contain the patient DNA samples with unknown genotypes for the β -Globin gene.

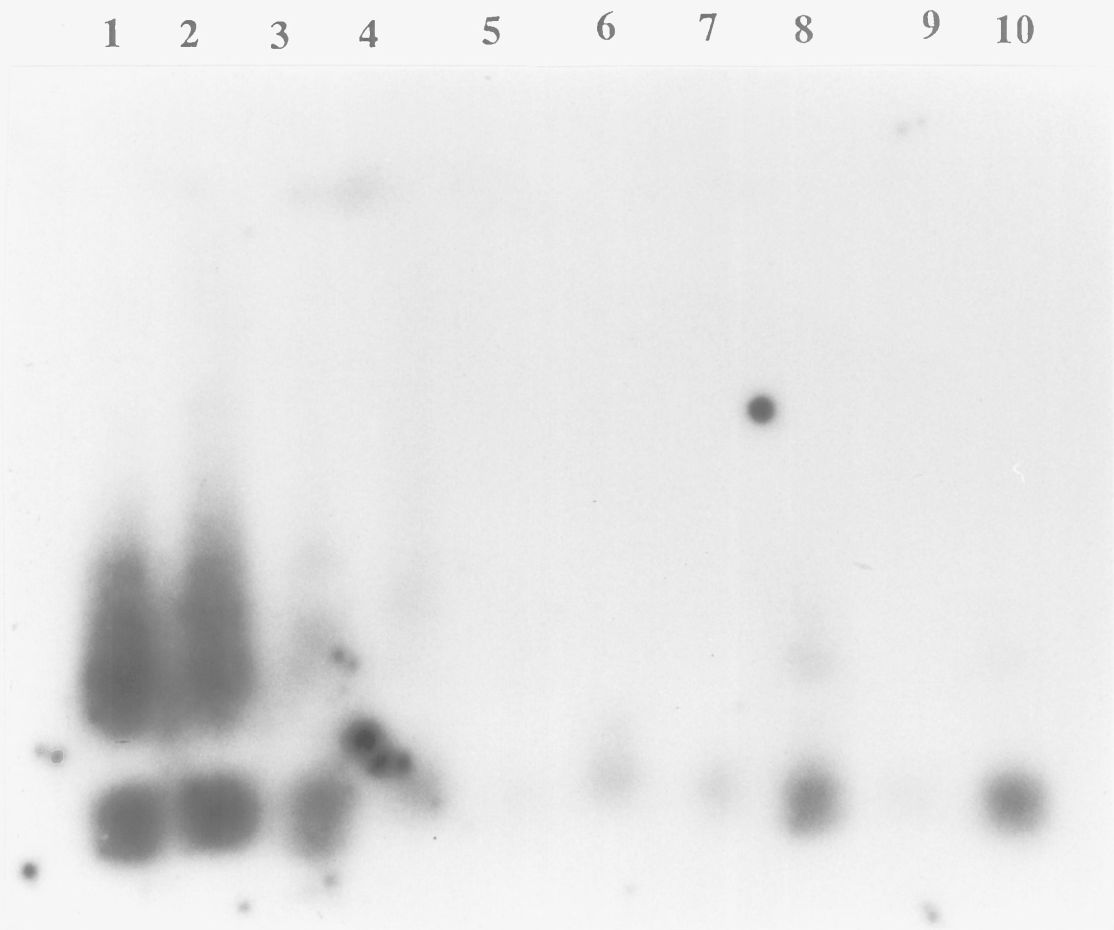


FIGURE 58 A DIRECT GEL HYBRIDISATION OF HUMAN PCR AMPLIFIED β -GLOBIN DNA WITH A 20MER OLIGONUCLEOTIDE PROBE SPECIFIC FOR THE NORMAL GENOTYPE AROUND THE SITE OF THE SICKLE MUTATION

Ten genotypically unknown samples of human DNA (0.5ug) were amplified using the PCO 3 and PCO 4 primers and were then subjected to electrophoresis through a 3% Nu Sieve agarose GTG/1% normal agarose gel. Once denatured and dried into the gel matrix, the immobilised DNA samples were hybridised with 10 pmol radioactively labelled 20mer oligonucleotide. Stringent post hybridisation washes could clearly differentiate between the genotypes present. Lanes 1-3 and 8+10 contain DNA with the sickle genotype, lanes 4-7 and 9 contain DNA with the normal genotype. The variation in signal intensity is a reflection of the variation in PCR amplification.

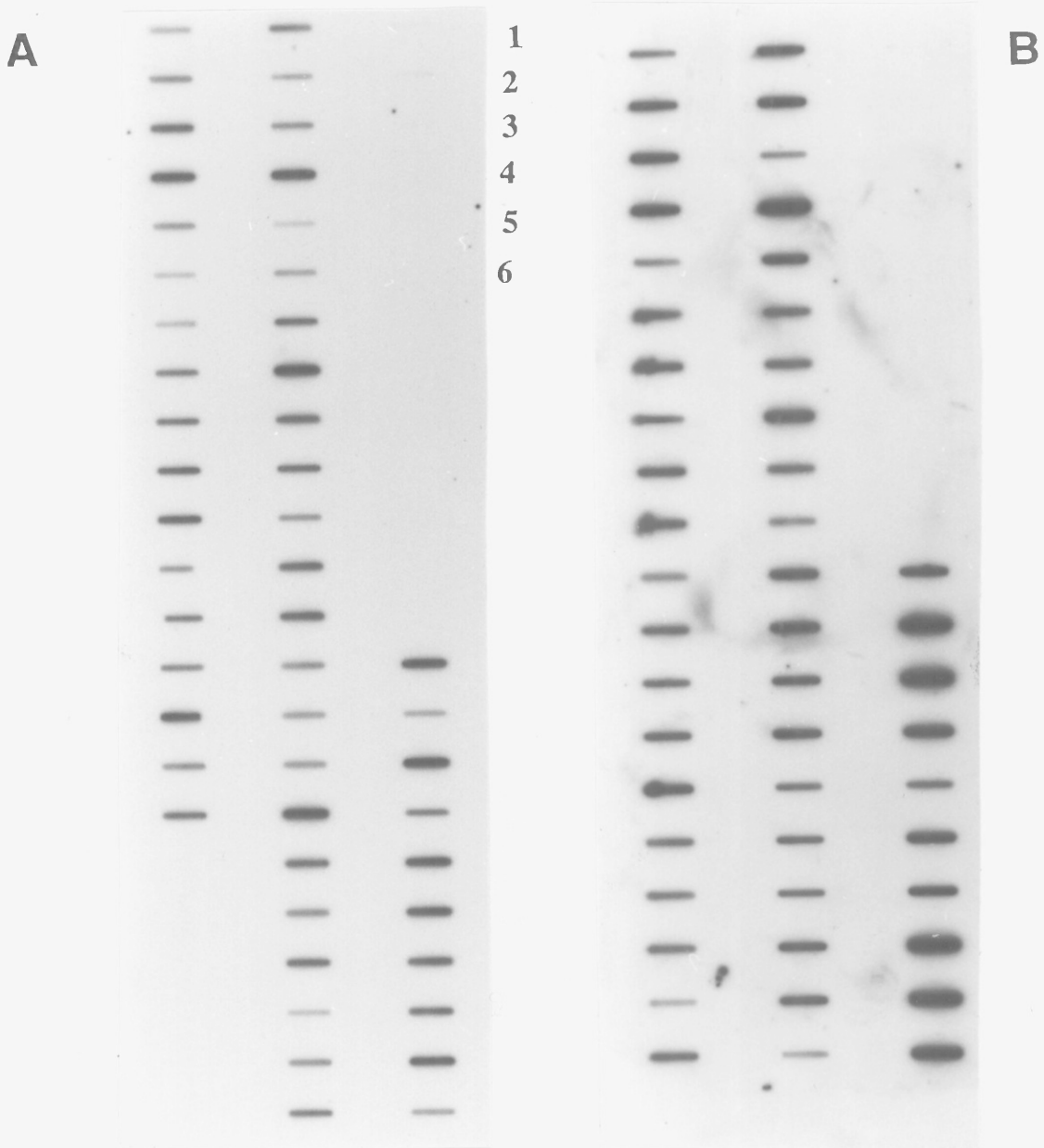


FIGURE 59 SLOT-BLOTS OF 50 PCR AMPLIFIED PATIENT DNA SAMPLES,AMPLIFIED AROUND THE SICKLE MUTATION SITE AND HYBRIDISED UNDER STRINGENT CONDITIONS WITH EITHER THE SICKLE OR THE GLOBIN 20MER OLIGONUCLEOTIDE PROBE

One fifth of each PCR amplified reaction was applied to nitrocellulose through slot-blotting.Each sample was denatured using 5M NaOH and neutralised using ammonium acetate.The filters were then hybridised with 20 pmol of ^{32} P kinase end-labelled 20mer oligonucleotide.Filter A. contains the results obtained using the sickle probe and filter B used the normal (analogue containing) globin probe.Stringent post hybridisation washing manipulations were carried out at 60⁰ C,however successful differentiation between the three genotypes was not achieved.The meaning of this is discussed in the text.Exposure of the filters to film was not sufficient to clearly detect the marker DNA samples in lane 1-6 (20,10,5,1,0.5,0.1 fmol) , a serial dilution of recombinant DNA markers for the globin gene.This suggested that the concentration of patient DNA in each well was more than 20 fmol.

genotype. These reoccurring bands did not appear to be complementary to the beta-globin 20mer oligonucleotide probe. These bands might have been caused by repeated hybridisation of the amplification primers to slightly mismatched sequences. Such amplifications would probably have been encouraged by the rapid activity of the polymerase enzyme, stabilising mismatched duplexes through chain elongation.

³²P Kinase end-labelled sickle and globin (analogue containing) 20mer oligonucleotide probes were used for slot-blot hybridisation reactions, to the PCR amplified human samples. The genotypes established from these hybridisations were found to be incorrect for a large number of the samples (errors of 44%). The probes used did not appear to be differentially hybridised to the nitrocellulose bound samples (figure 59). This may have been caused by the method sample application, which involved suction to the filter under vacuum. Such procedures may have made a considerable amount of DNA unavailable for hybridisation. Alternatively the sample wells may have been overloaded with PCR amplified DNA. As a result differential washing manipulations may not have affected the sample wells equally.

3.8 Sandwich hybridisation using human DNA samples

To test whether the sandwich assay could be used with amplified target DNA, a series of hybridisation reactions were carried out. These reactions were performed using the 100mer oligonucleotide capture probe immobilised through diazotization to Sephacryl S-1000. The target DNA used in these reactions was the 110bp beta-globin fragment generated through PCR amplification of human DNA (as described earlier). The hybridisation efficiencies obtained were compared with those found using recombinant beta-globin gene fragments (208bp). To establish the degree of non-specific binding, non-complementary target,

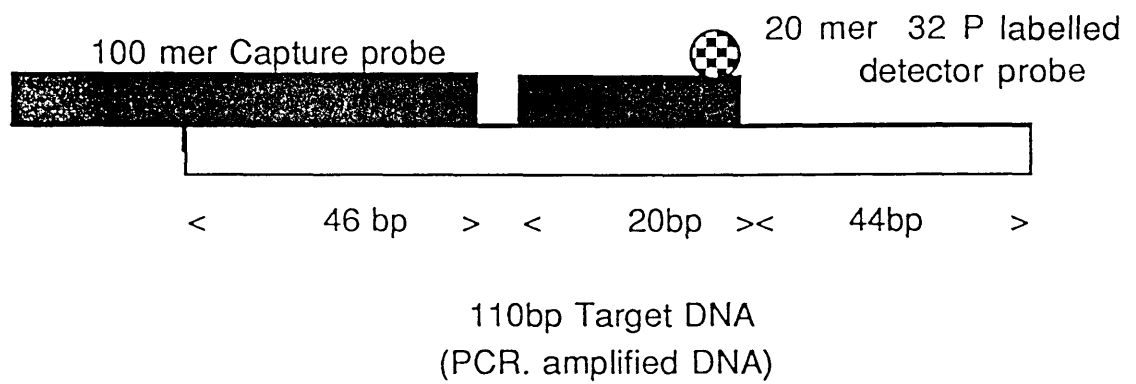


**FIGURE:60 SANDWICH ASSAY USING HUMAN AMPLIFIED
TARGET DNA AND TWO OLIGONUCLEOTIDE PROBES**

detector probes and resins without immobilised capture probe were used. An outline of the sandwich hybridisation strategy used is shown in figure 60.

3.8.1 Hybridisations with known concentrations of human DNA

Sandwich hybridisations using 0.5 pmol capture probe, 10 fmol amplified DNA and 0.5 pmol detector probe were performed. These DNA concentrations were chosen as they had previously been found to suit efficient sandwich hybridisation. The PCR amplified patient DNA samples used were of the homozygous normal and sickle mutated genotypes of the beta-globin gene. The amplified DNA samples were purified from smaller DNAs through "Nensorb-20" purification and were diluted to femtomolar concentrations, before use in the sandwich assay. The dilution factor was estimated from absorbance readings of the Nensorb purified DNA samples at O.D 260 nm.

Unamplified target DNA, derived from the recombinant plasmids Hbf-5, Hbl-S, was used in control hybridisations. This enabled the better characterisation of hybridisation efficiencies, of amplified target DNA. Other controls for these hybridisation reactions included the use of non-complementary target DNA fragments of 1600bp. Previous data suggested that reliable non-specific binding results could still be obtained, despite the considerable size difference between control and amplified target DNA (1490bp),

The hybridisation results obtained using PCR amplified human DNA, were found not to differ significantly from those found using the recombinant DNAs. Hybridisation efficiencies were determined by assuming 100% binding of the detector probe when all of the target DNA was involved in duplex formation. The two genotypes of the PCR amplified DNA samples used in these experiments could be clearly

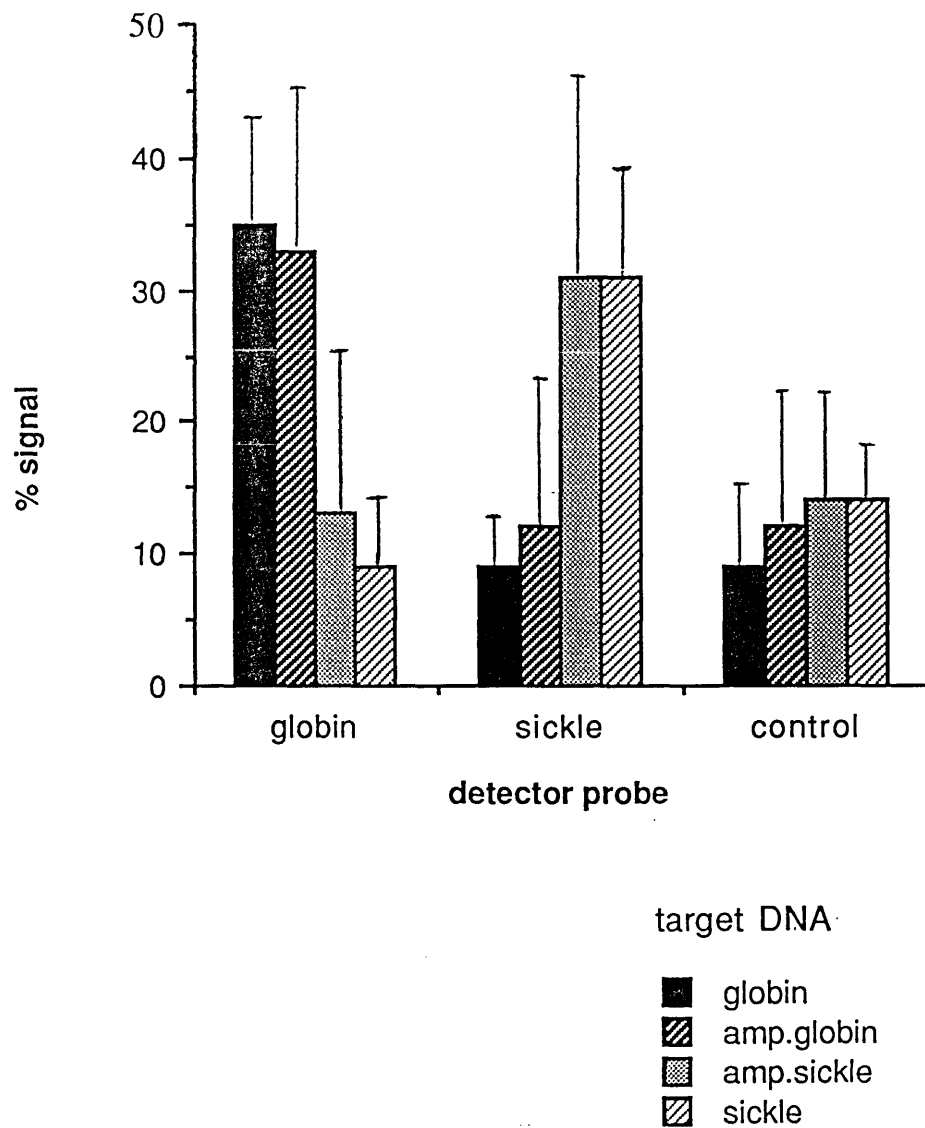


FIGURE 61 SANDWICH-HYBRIDISATION USING SAMPLES OF HUMAN DNA AMPLIFIED AROUND THE SICKLE MUTATION SITE BY PCR

The hybridisations were performed with the 100mer beta-globin specific capture probe immobilised by diazotization (500 fmol), the 110 bp target DNA, used at a concentration of approximately 10 fmol (determined by A260 nm readings), and 20mer (32 P kinase end-labelled) detector probes. Normal, sickle and control 20mer oligonucleotides complementary in sequence to the target DNA were used as detector probes. Hybridisations were left to incubate overnight at 50°C. The results show that the matched duplexes hybridised two times more efficiently than mismatched sequences. The control hybridisations shown were performed with non-complementary 20mer detector probes. These non-specific attachments were similar to those recorded for the controls carried out without the capture and target probes ($6.5\% \pm 5$ and $8\% \pm 4$ respectively).

distinguished from each other. A two to four fold difference in hybridisation of the detector probe was recorded, between matched and mismatched hybrid duplexes.

3.8.2 Hybridisations with unknown concentrations of human DNA

A sandwich hybridisation study, with 25 PCR amplified samples of human DNA, with unknown beta-globin genotypes, was performed. The concentrations of the three sandwich components were; 20 fmol capture probe (100mer immobilised by diazotization), approximately 1 pmol target (5 ul of amplified sample), 60 fmol detector probe.

Previous sandwich hybridisation data with recombinant DNA samples had suggested that increased target concentrations would result in increased hybridisation. Stringent washing manipulations were however required to remove the increased percentage of non-specifically bound target DNA. The concentrations used were chosen primarily to minimise the effect differences in target amplification might have on the assay. This ratio would also eliminate the need for sample dilution, thus removing one step from the assay, and reducing the risk of target denaturation.

The detector probes used in this analysis were the 20mer globin oligonucleotide, containing the analogue 2-aminoadenine, and the 20mer sickle oligonucleotide. Control hybridisations included reactions carried out with recombinant beta-globin target DNA and hybridisations omitting target DNA or resin immobilised capture probe.

The hybridisation protocol used, included a post-hybridisation wash for 1 hour at 50°C, followed by a 30 minute wash at 60°C. After each washing step, the resin bound signals were measured through Cerenkov counting. These manipulations enabled differentiation between the two genotypes of the recombinant samples used. The hybridisations

efficiencies found for these samples were then used to establish the genotypes of the unknown PCR amplified human samples. The results are listed in table 20. Matched hybridisations of the normal probe were recognised as being at least 2 times greater than the mismatched hybridisation efficiencies found using the recombinant target DNA.

Using this criterion, all of the genotypes were correctly determined from the hybridisations with the analogue containing probe. Heterozygous and homozygous normal genotypes for the beta-globin gene both displayed hybridisation efficiencies above 12%. The mean differences in hybridisation efficiency between the three genotypes, measured with the analogue containing probe, were however not great. The mean percentages obtained for each were recorded as; 18% for the homozygous normal, 14% for the heterozygous, and 9% for the homozygous abnormal genotype.

Using the sickle detector probe sample genotypes were not easily determined. The comparison of the experimentally found genotypes with the actual genotypes revealed an error of 20%. This error was probably caused by the extensive and stringent washing manipulations, required to reduce non-specific bindings. Better results may have been obtained with a lower concentration of target DNA.

From the results, displayed in table 20, it became clear that the analogue containing oligonucleotide was better suited for this sandwich assay, primarily because of its increased stability when duplexed to complementary DNA.

Improvements were still required to differentiate more clearly between the signals obtained from genotypically heterozygous and homozygous abnormal samples. The variation in hybridisation efficiencies found for both probes, may have in part been a reflection of the variation in DNA amplification. This variation in amplification appeared to present a problem with respect to differential

Sample nr.	% hybridisation		diagnosed	actual
	a)	b)	genotype	genotype
1.	15	9	+ - S	sickle
2.	3	15	- + G	globin
3.	14	10	+ - S	sickle
4.	5	18	- + G	globin
5.	9	10	+ - S	sickle
6.	14	7	+ - S	sickle
7.	4	16	- + G	globin
8.	9	9	+ - S	sickle
9.	7	8	- - ?	sickle
10.	5	12	- + G	heterozygote
11.	7	15	- + G	heterozygote
12.	6	16	- + G	heterozygote
13.	5	22	- + G	globin
14.	10	9	+ - S	sickle
15.	8	15	+ + H	heterozygote
16.	10	14	+ + H	heterozygote
17.	14	10	+ - S	sickle
18.	12	10	+ - S	sickle
19.	4	16	- + G	globin
20.	5	29	- + G	globin
21.	4	8	- - ?	sickle
22.	3	13	- + G	globin
23.	14	8	+ - S	sickle
24.	8	12	+ + H	heterozygote
25.	8	12	+ + H	heterozygote
globin control	16	6	2 x 6 =	positive signal
sickle control	13	4	2 x 4 =	positive signal
control - resin	3			
control - target	9			

TABLE 20 SANDWICH-HYBRIDISATION ASSAY USING GENOTYPICALLY UNKNOWN BETA-GLOBIN TARGET DNA'S AND UNKNOWN CONCENTRATIONS OF TARGET DNA, PCR AMPLIFIED TO GIVE 110BP FRAGMENTS OF SEQUENCES CODING FOR THE REGION AROUND THE SICKLE MUTATION SITE.

The hybridisations were performed with 25fmol 100mer immobilised capture probe, immobilised by diazotization, 60 fmol detector probe and 1 ul target DNA sequence, taken directly from the PCR reaction of each human sample. The hybridisations were incubated overnight at 50°C in a total reaction volume of 110 ul. To ensure stringent washing conditions the resins were washed at 63°C upon hybridisation. Column a) lists the hybridisation results obtained using the 20mer sickle detector probe and column b), lists those recorded using the normal globin detector probe, containing the 2-aminoadenine analogue. The values highlighted by bold print correspond to the errors made in determining genotypes. Most errors are due to poor hybridisation efficiencies with the sickle probe. This may be a direct result of the stringent washing conditions. The results also show that not all target DNA samples hybridised with the expected efficiencies, suggesting that there had been considerable variation in target DNA amplification.

hybridisation to the three genotypes.

3.8.3 Summary

1. The two homozygous genotypes for the beta-globin gene could be clearly distinguished from one another through a two-fold difference in detector probe hybridisation.
2. A 2 fold differentiation between duplex formation of the heterozygous genotype and the mismatched duplexes was not obtained, with the sandwich hybridisation procedures used.
3. Extensive washing manipulations (required to remove excess target DNA), were associated with a decrease in the retention of matched duplexes, with normal detector probes.
4. Detector probes containing the 2-aminoadenine analogue, formed duplexes more stable to extensive washing manipulations at elevated temperatures than normal probes.
5. Variation in target amplifications may have affected the efficiency of oligonucleotide probe hybridisations in the sandwich assay.

3.9 Differential hybridisations using non-radioactive probes

Using recent methods available for labelling oligonucleotides, experiments were carried out to establish the ease with which the sandwich assay could be converted to a non-radioactive assay. Apart

from the increased safety aspect, the non-radioactive label would provide a more rapid detection system. Most detection strategies could generally be carried out within a few minutes.

Recent data by Urdea et al (1988) had suggested that enzyme modified oligonucleotides could be used in hybridisation assays, providing sensitivities comparable to ^{32}P labelled probes. The method of incorporating these labels was via an alkylamino derivative, which could be added during synthesis of the oligonucleotide. The more ideal labelling strategy was found to be one using horseradish peroxidase (HRP), detected colorimetrically or through enhanced luminescent techniques. The detection limits of these probes used in hybridisation experiments were established as being around 0.1 fmol. This limit was defined as the minimum quantity of analyte, to give a signal to background (noise) ratio of two. Using ^{32}P kinase end-labelled oligonucleotides in comparison resulted in a detection limit around 0.05 fmol.

Using the protocols recommended by Urdea et al (1988), alkylamino oligonucleotides were synthesised, some of which were derivatised with HRP and some with biotin. The rationale behind choosing biotin as an alternative labelling method was that it could be used as an indirect enzymatic label. Detection of the biotin derivatised oligonucleotides could be achieved by reacting the probes, with an HRP-streptavidine conjugate, at room temperature. The enzyme would in this way avoid being exposed to the stringent hybridisation and washing conditions required of the labelled detector probe during sandwich hybridisation.

The oligonucleotides used for these experiments were beta-globin specific 20mers, coding for both the normal and the sickle mutated gene sequence.

3.9.1 HRP probe filter hybridisations

Enzyme labelled probes were prepared by a procedure directly linking HRP to a 5' alkylamino group, as described in the material and methods section. Unincorporated HRP was removed from the labelled and unlabelled oligonucleotide probes through affinity chromatography.

In order to determine the detection limits of enzyme modified oligonucleotide probe a series of dilution experiments were performed. The detection limits of both purified and unpurified oligonucleotide probe were compared.

A constant amount of o-phenylenediamine (OPD), water soluble dye and hydrogenperoxide substrate were used for colorimetric detection of the HRP label. The relationship between probe concentration and O.D. 490 nm absorbance was that expected of a typical enzyme reaction, not limited by substrate concentration, as depicted in figure 62. From the results, concentrations of approximately 0.35 fmol were still detected after purification of the labelled oligonucleotide.

Dilutions of the HRP enzyme itself were also assayed to ensure that linkage to the oligonucleotide did not alter enzyme activity. No apparent loss in enzyme activity was observed upon linkage.

When insoluble reagents for colorimetric detection were examined such as 3,3'-dianisidine, a precipitate could be detected. The detection limits, however, appeared to be considerably less than those found using the soluble reagents.

To determine the sensitivity of the HRP labelled probes upon hybridisation, dot-blot experiments with complementary plasmid DNAs were performed. Enzyme restricted, recombinant beta-globin DNA was applied to nitrocellulose filters in dilutions of 20, 10, 5, 1, 0.5, and 0.1 femtomoles. Dilutions of HRP were also applied to strips of nitrocellulose. The detector probe was used 150 times in excess over

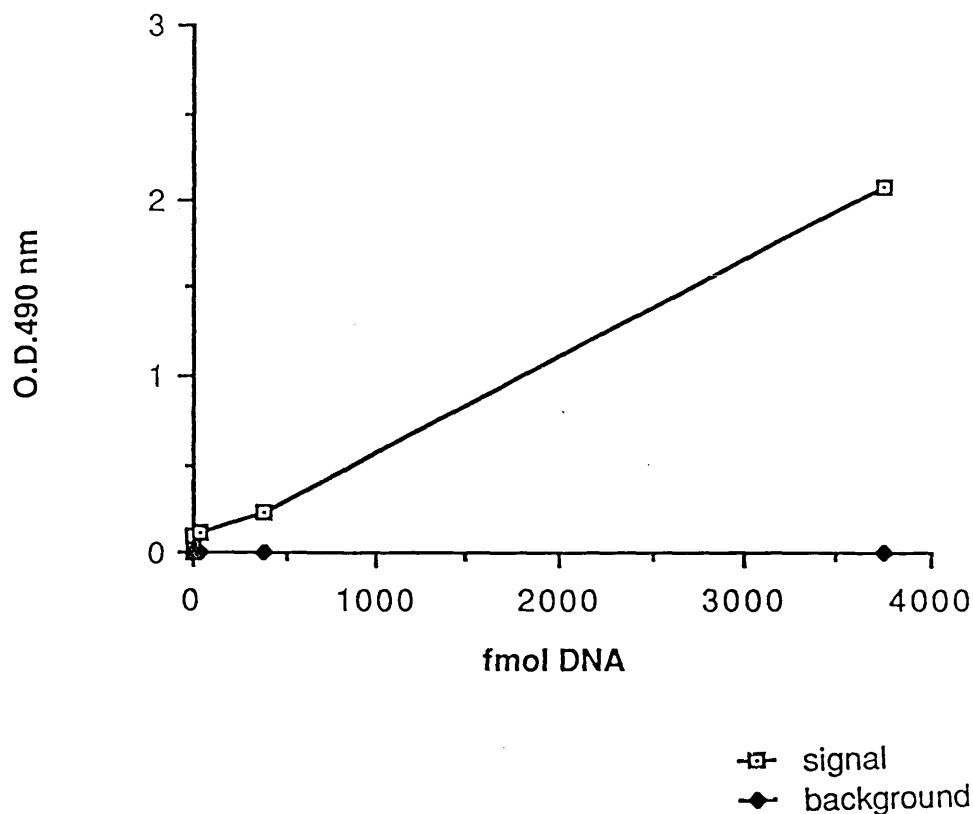


FIGURE 62 THE RELATIONSHIP BETWEEN THE AMOUNT OF HRP LABELLED OLIGONUCLEOTIDE AND THE O.D.490 NM OF THE OPD REACTION PRODUCTS

The signal observed was obtained using purified 20mer oligonucleotide probe end-labelled with HRP (having removed all unincorporated HRP molecules). To each dilution of labelled oligonucleotide, 100ul OPD solution was added. The samples were incubated for 20min at 37°C and the colourimetric reactions were terminated with H₂SO₄. Optical densities were measured as described in 2.3.12.2 of the methods section. The DNA concentrations were estimated from the initial input concentrations measured prior to end-labelling with horseradish peroxidase. The estimates assumed that no further DNA loss had occurred during purification of the end-labelled oligonucleotide.

the total target DNA present in each hybridisation reaction.

Hybridisations to the target DNA were performed at 38°C in the presence of 17.8 formamide (equivalent to a hybridisation temperature of 50°C). Other conditions used were those normally applied to filter hybridisations, described in the materials and methods section. Stringent washing steps were carried out on a selection of the filters prior to detection with the insoluble colour reagent 3,3'-dianisidine. One of these steps included washes with formamide buffer, equivalent to stringent washes at 60°C. Some of the filters were left unwashed.

When the filters were stained with the insoluble dye complex, according to the methods by Renz et al (1984), no oligonucleotide probes could be detected. Non-specific binding did not appear to be the limiting factor with respect to probe detection, as no significant background was produced. When the HRP filters, which had not been exposed to hybridisation were assayed in the presence of the same dye complex, no probe was detected. Exposing the filters to the soluble dye OPD, however could detect HRP labelled probe. The soluble dye colour formed, diffused slowly from the applied dots, showing that a significant signal over background could be obtained with OPD.

Using another insoluble dye, 3,3',5,5'-tetramethylbenzidine was used in similar filter hybridisation experiments, the same results were obtained. This suggested that both of the insoluble dyes required a minimum concentration of HRP label for probe detection. Perhaps a greater concentration of enzyme incorporated into the probe would have formed a precipitate which could be detected by eye.

3.9.2 Biotin probe filter hybridisations

Long chain N-hydroxysuccinimidyl biotin moieties were linked to the 5' alkylamino groups of oligonucleotide detector probes. These were

purified in the same fashion as the enzyme modified probes.

Colorimetric detection was achieved by reacting a streptavidine-horseradish peroxidase complex with the biotin labelled probe. This was because streptavidine is known to have a strong and highly specific binding affinity towards biotin. The detection limits of the enzyme complex were not assayed.

Dot-blots were carried out using an excess of the biotin detector probe and complementary cloned beta-globin sequences, applied to nitrocellulose in concentrations of 20, 10, 5, and 1 fmol. The hybridisations were carried out under the stringent conditions found to suit differential hybridisation. Upon hybridisation the filters were exposed to the streptavidine complex as described in materials and methods. Colorimetric detection was carried out using the insoluble dyes 3,3'-dianisidine and 3,3',5,5'-tetramethylbenzidine. No detection of the hybridised detector probe was achieved by these methods.

These results supported the view that it was not heat denaturation during hybridisation, but a limited availability of HRP enzyme, which limited detection of the non-radioactively labelled probe.

3.9.3 Sandwich hybridisation with non-radioactively labelled probes

Sandwich hybridisation assays were conducted with 250 fmol immobilised 100mer capture probe, and 5 fmol, 208 bp recombinant beta-globin target DNA. The two DNAs were hybridised at 65°C and for one hour, in a one-step hybridisation reaction, under stringent conditions. Following a series of washes, approximately 150 femtomoles of the HRP end-labelled beta-globin specific detector probe (normal or sickle 20mer probe) was added to the reactions and left at room temperature for 5 minutes. A control without target DNA was run in

duplicate with each hybridisation reaction. A number of stringent washes were carried out to ensure removal of the unhybridised detector probe, prior to colorimetric detection of the enzyme label with O.P.D.. The absorbance readings of the dye colour released were taken from supernatant of each reaction, transferred to a microtitre dish well.

The results were disappointing, as each reaction displayed a dark colour reaction of equal intensity, including the controls. A considerable amount of the colour was also found to be quenched by the resin, suggesting that non-specific binding of the detector probe was occurring as well as direct adsorption of the dye.

A reduction in the molar concentration of the detector probe used over the target DNA concentration, might have reduced the non-specific attachments. Furthermore, increasing the concentration of blocking agents in the hybridisation solution might also have reduced some of the non-specific background. Alternatively non-specific attachments might have been reduced by using a different resin support matrix. It seemed unlikely that carrying out the assay in a one-step reaction, with all the DNA components present at once, would diminish the interference of non-specific binding.

From the results obtained it appeared that the use of non-radioactively labelled detector probes, in the sandwich assay developed for ^{32}P was not immediately possible. The main conclusion from these experiments was that enzyme modified probes could probably be used in the sandwich hybridisation assay, if the support matrix were suitably coated with effective blocking agents, or a different support matrix such as polystyrene, was used.

3.9.4 Summary

1. The water soluble colour reagent o-phenylenediamine (OPD) could be used to resolve attomolar concentrations of free HRP end-labelled oligonucleotide.
2. Insoluble dyes such as 3,3'-dianisidine and 3,3',5,5'-tetramethylbenzidine could not be used in filter hybridisations to detect HRP end-labelled oligonucleotide probes. The reason for this was probably the limited incorporation of enzyme into each oligonucleotide probe.
3. Non-specific background binding of the HRP end-labelled oligonucleotide detector probes to the Sephacryl support made it difficult to perform sandwich hybridisation with these probes.

4.

DISCUSSION

The development of a sandwich hybridisation assay for the detection of single base changes in genomic DNA has resulted in several interesting observations. These can be grouped into four categories: the choice of reagents, hybridisation conditions, hybridisation kinetics and the sensitivity of the assay developed. These categories are considered with respect to the advantages and disadvantages of the studied assay configuration.

a) The choice of reagents

Almost immediately, it became apparent that the choice of reagents and immobilisation strategy were crucial for the success of an oligonucleotide based sandwich assay. Factors examined in choosing a suitable coupling chemistry for oligonucleotide immobilisation included, the coupling yields, the magnitude of non-specific binding, the availability of attached oligonucleotide to hybridisation, and the stability of hybrid duplexes formed with the immobilised sequence.

Carbodiimide mediated immobilisation was quickly discarded as being an unsuitable chemistry, primarily for the poor availability of immobilised DNA to hybridisation (11-23%). Also long recombinant fragments immobilised by this method were associated with poor hybridisation efficiencies to complementary target DNA.

Recent data by Gingeras et al. (1987) reported findings of equally low hybridisation efficiencies with oligonucleotides end-immobilised using carbodiimide (27%). They suggested that these could, however, be improved by pre-hybridising the support bound oligonucleotides with dextran sulphate. This pre-hybridisation was thought to affect the reaction in the following two ways. One function

of the dextran sulphate was to encourage full extension of the support bound oligonucleotide into the solution, by disrupting the adsorptive forces holding the oligonucleotide to the surface. The other function was to decrease the apparent reaction volume by excluding the nucleic acids from the volume occupied by the dextran sulphate.

Dextran sulphate could not be used, in the same way, for a sandwich hybridisation. This macromolecule appeared to act in a disruptive fashion on duplex formation, decreasing the hybridisation efficiencies of detector probe. This effect was first reported by Ranki et al (1983), and confirmed in the following study (data not shown).

Initial work suggested that the adapted method of enzymic immobilisation would suit sandwich hybridisation. High coupling yields could be achieved, with oligonucleotides available for specific hybridisation to target sequences, at high efficiencies. It was not until the third approach to immobilisation was examined that the disadvantages of this immobilisation strategy became apparent.

Oligonucleotide capture probes appeared to form less stable hybrid complexes with target DNAs than probes immobilised by diazotization. Vigorous washing manipulations destabilised a high percentage of the sequence specific duplexes formed between enzymically immobilised probe and target, as well as the nonspecific attachments. This was similar to the unexpected losses encountered with end-linked capture probes, bound using carbodiimide (CMC).

Hybridisation reactions with the CMC bound probes were found to be associated with a 47% target loss between the second and fourth post-hybridisation wash. A 26% loss for the same hybridisation reaction between identical probe and target lengths was recorded for oligonucleotides immobilised by diazotization. Some of this loss would have been expected to occur as a result of stringent washing

manipulations and the removal of non-specifically bound target. Another part could be accounted for from chemical instability of the oligonucleotide probe to the resin support. These losses could however not entirely account for the hybrid instabilities recorded for end-linked oligonucleotide probes.

Such hybrid instabilities could perhaps be explained by an increased strand displacement activity of the target DNA. Duplex formation with capture probe, extended from the resin support, might encourage target rehybridisation. Alternatively, target DNA hybridised to diazotized capture probe might form hybrids of increased stability as a result of adsorption to the solid support. Any single-stranded overlap in target DNA might be stabilised through such adsorption, whereas in the unbound state such strands could act disruptively, forcing the duplex to dissociate.

Disruptive forces acting on hybrid duplexes and generated from overlapping single stranded target might account for the loss in enzymic immobilisation between a 20mer and a 50mer oligonucleotide. A 29% drop in immobilisation efficiency was recorded.

Another interesting observation concerns the effect increasing capture probe length had on hybridisation, with end-linked, and random base immobilised sequences. Increasing capture probe lengths from 20 to 100 base pairs had little effect on the hybrid stability of end-linked DNA duplexed with different target lengths. Increasing the length of capture probes immobilised by diazotization however had a marked effect. Hybridisation efficiencies were enhanced with increasing capture probe lengths.

It would seem possible that the decrease in guanine content of the capture probes used, could account for this increase, as suggested previously in section 3.5.3. Guanine moieties have been shown to immobilise in preference to other bases when exposed to diazotization

(Noyes and Stark, 1975). A decrease in the number of base attachments might have resulted in an increase in hybridisation efficiency. Increases in hybrid stability of the diazotized probe could also have been attributed to a decrease in the disruptive forces, mentioned previously.

The detector probe suiting differential hybridisation between normal and single base mutated target DNA was found to be the probe containing the analogue 2-aminoadenine. Increased hybrid stability was brought about by the presence of an additional hydrogen bond between the 2-aminogroup on the purine ring, and the 2-oxo group on the pyrimidine ring of the complementary thymine. The same hydrogen bonding site generated decreased hybrid stability when hybridised to a mismatched base.

Increased hybrid stability with the probe containing analogue, was characterised by an increase in melting temperature of the matched but not the mismatched duplex. This property enabled a clearer differentiation between the hybridisation of detector probe to matched and mismatched sequences. Ideally this assay would be performed using only such detector probes. In the present study only one probe containing analogue was available.

b) Hybridisation conditions

The sandwich hybridisation conditions for oligonucleotide probes were found to differ in certain fundamental aspects to those reported using only recombinant DNAs. The affinity capture and characterisation of single stranded target DNA required hybridisation temperatures which suited both detector and capture probes and were usually below 65°C. This temperature dependance could however be eliminated by using oligonucleotide probes of increased lengths.

Oligonucleotide length for differential hybridisation was however limited, as differences in hybrid sequence were characterised by differences in melting temperature of the detector probe. Unless a base analogue was found which fundamentally altered the optimum hybridisation temperature of a 20mer probe, without affecting its melting properties, the temperatures used in the assay developed, would always be determined by the detector probe.

Two-step hybridisations, splitting the reaction events of target and detector probe and using the temperatures suiting the hybridisation of each sequence, did not seem to affect the final efficiencies recorded.

The prime disadvantage of this temperature dependance is that low hybridisation temperatures could encourage the non-specific binding of target sequences. Such binding events would decrease the sandwich hybridisation efficiencies, observed in this assay.

One expected observation found using oligonucleotide probes in a sandwich assay, was the increase in non-specific binding of the detector probe to the resin support. The increase in non-specific binding could be anything from 50% to 60% greater than the background generated using recombinant DNAs. This property was initially demonstrated to be a significant problem when filter hybridisations to immobilised target were performed with labelled oligonucleotides. Prior to the post hybridisation washes, oligonucleotides appeared to hybridise efficiently at a temperature 5°C below the optimum hybridisation temperature. Upon washing the filters, however, a large percentage of the signal obtained was found to be the result of non-specific binding events.

Non-specific binding of oligonucleotides could be reduced through effective blocking of the support surfaces with non-complementary oligonucleotide sequences and using other

macromolecules such as sonicated, denatured, salmon sperm DNA.

To maximise target detection, probe concentrations are generally chosen to be in excess, thus driving the reaction and discouraging reannealing of denatured target DNAs. Results, however, demonstrated that a vast excess of detector probe was not required in a sandwich assay for efficient target DNA detection. Although increased detector probe concentrations resulted in an increase in the signal bound to the resin, increased background binding was also found to occur.

The reason for this affinity of oligonucleotide probes to the surfaces of resin supports was unclear. Ultimately the only effective way of reducing this binding would be to use a different support system with lower binding affinities for oligonucleotides.

c) Hybridisation kinetics

In experiments performed with different concentrations of end-labelled target DNA, hybridisations with increased target DNA concentrations appeared to result in increased hybridisation efficiencies. Whether the kinetics of hybridisation were also increased with increased target concentrations could not be determined from the data obtained. Maximum hybridisation of the target was generally achieved in 30 minutes. Maximum saturation in a sandwich assay, of the capture probe with hybridised target and detector probe, was reached in the slightly slower time of one hour.

These hybridisation times were a significant improvement over the times reported for sandwich hybridisations performed with recombinant fragments. In these experiments overnight incubations were reported necessary for maximum hybridisation (Langdale and Malcolm, 1985). It would therefore seem that oligonucleotide hybridisations are more rapid, a property expected of shorter nucleotide sequences.

Using excess target DNA in the sandwich assay performed with unknown concentrations of PCR amplified genomic DNA, it was hoped to maintain the kinetic driving force of hybridisation. From the results observed this may have been achieved. However, increased target concentrations were also associated with increased background. Some of this background could perhaps have been avoided, had an increased concentration of resin immobilised capture probe been used. Further work in optimising the sandwich assay for such analysis is required.

d) Sensitivity of the sandwich hybridisation assay

From simple target hybridisation experiments, to immobilised capture probe, it became apparent that the sensitivities of the reaction would not be sufficient to detect single copy genes. The practical limits of target detection were found to be around 1 fmol. From filter hybridisation data, it became clear that the detection limit of oligonucleotides was primarily caused by the limitations in incorporating sufficient radioactivity, in the nucleotide sequence. PCR amplification of the target DNA provided a solution to this problem, by increasing the single copy gene fragments being analysed from attomolar to femtomolar concentrations.

Initial observations suggested that the sandwich assay developed could easily accommodate the use of PCR amplified target DNA. The study carried out using unknown concentrations of PCR amplified target DNA outlined some of the difficulties which could be encountered in using this assay for routine diagnosis.

The efficiency of PCR amplification was not reliable and did not always amplify target DNAs to expected concentrations. Furthermore the amount of product formed could not be determined easily. To overcome these difficulties several amplification reactions using the

same target DNA would be required. Samples of each reaction would then have to be incorporated in separate hybridisation reactions, before a reliable determination of target genotype could be made. By performing control amplification reactions, incorporating radioactively labelled nucleotides in the last cycle of amplification, one might be able to estimate the amount of product formed. Using target samples of similar concentrations measured in this way, might help to standardise the assay developed.

Another observation which could limit the success of efficient sandwich hybridisation using PCR amplified target DNA, was the degree of non-specific amplification. By increasing the stringency of conditions required for primer annealing most of these non-specific amplifications could be prevented.

Finally but most importantly enzyme mediated PCR amplification is known to incorporate sequence errors during DNA strand synthesis. A recent finding on the fidelity of DNA synthesis by the Thermus aquaticus DNA polymerase, suggested that this enzyme produced single-base substitution errors at a rate of 1 for each 9000 nucleotides polymerised. Frameshift mutations were also found to be produced, at a frequency of 1/41,000. The frequency of single-base substitutions generated with the large Klenow fragment of DNA polymerase I on the other hand was considerably lessened by the active 3'-5' proofreading exonuclease associated with this enzyme. Error rates were estimated to be around 1 base change in every 40 000 nucleotides polymerised (Tindall and Kunkel, 1988).

Heat treatment of the Taq polymerase enzymes was likely to increase the mutations occurring during PCR amplification. Heat treatment of DNA has also been reported to be mutagenic, generating both transitions and transversions (Tindall and Kunkel, 1988 and references cited therein). Because the target sequence is amplified

exponentially during PCR amplification, even small sequence errors may become a significant proportion of the reaction product, depending on how early in amplification this error occurred. To minimise the heat induced damage of DNA and replication errors of DNA synthesis, during PCR amplification, one should consider the use of reaction conditions limiting incubation times at higher temperatures.

For single base change detection using the sandwich assay developed, one could go one step further and reduce the number of cycles used to PCR amplify samples of DNA. Theoretically sixteen cycles would be sufficient to PCR amplify 0.1 attomoles of single copy gene (present in 200 ng of genomic DNA, from 20 μ l blood) to femtomolar concentrations (6.6 fmol). This would in turn permit several sandwich hybridisation assays to be performed with optimal concentrations of target DNA. Such measures would minimise the effects of mutation arising during PCR amplification. By duplicating or even triplicating each assay one could further reduce the chances of mis-diagnosis due to PCR induced mutational error.

In spite of this potential error, the probability of a single base change occurring within the 20 nucleotide target sequence to which the detector probe is complementary, is slight.

e) Advantages of the assay configuration

The use of oligonucleotides for solid phase capture and target detection in a sandwich assay, has circumvented many of the problems previously associated with the use of recombinant DNA probes. Since automated synthesis of oligonucleotide probes is possible, large quantities of probe can rapidly be produced for little cost and effort. As the assay method described requires only femtomolar concentrations of each probe the actual cost of each assay is minimal.

(one synthesis, as described in the materials and methods section, would be sufficient for 3.8×10^6 assays).

The fact that oligonucleotides are single stranded, also provides a more accurate means of quantitative hybrid analysis. It has been suggested that (double stranded) denatured recombinant detector probes can reanneal in short stretches, generating a complex multimer of probe. The hybridised signal obtained from such a complex would then no longer be a quantitative measure of the target DNA present in the assay.

Furthermore, the kinetic properties of oligonucleotides reduce the required time of the assay from an overnight incubation, to one lasting only an hour.

One additional advantage of using oligonucleotides probes is that their specific hybridisation properties can be used, as shown in this study, to discriminate between target sequences with a single base change. Provided the base change for analysis and the surrounding sequence data is known, this assay could be applied to any genomic sequence.

As the basis of detection relies on PCR amplification of target DNA, only small samples of target DNA are initially required for this assay. These can be automatically amplified to large samples of target DNA in a short period of time (16 cycles would require a time of approximately one hour). Although purity and size of these fragments are difficult to control using PCR amplification, the products can still be utilised for reliable analysis using this sandwich assay. The possibility of using crude tissue samples for PCR amplification would enable this assay to be performed in under 3 hours, if all the required reagents were available. Also, it should be possible to perform several assays simultaneously, permitting the manipulation of large numbers of samples.

Recent work by Lench et al (1988) describes a technique of PCR amplification, which produces sufficient sample for direct gene analysis using DNA isolated from buccal epithelial cells, obtained by "mouthwash". This non-invasive approach to obtaining DNA for gene analysis would be much cheaper and simpler than using blood samples for screening of the carrier status in a number of genetic disorders. Pre-natal diagnosis should also be possible with small tissue samples obtained through amniocentesis or chorionic villus sampling (Tucker, 1988).

The disadvantage of a PCR based sandwich assay would be the high cost of the polymerase enzyme, particularly if the Taq polymerase enzyme was used. Alternative amplification strategies would have to be investigated in a cost effective sandwich assay system, such as the amplification multimer developed by Urdea et al (1987). This particular oligonucleotide multimer, however, has limited amplification properties (50-100 fold amplification), insufficient for the reliable analysis of single genes in small samples of genomic sequence.

The prime disadvantage of the assay system developed is the affinity of the resin support to non-specific binding of the oligonucleotide detector probe. Using oligonucleotide detector probes containing the analogue 2-aminoadenine, a considerable amount of this binding could be removed without affecting the stability of the sequence specific hybrids formed. An ideal sandwich hybridisation would require, either a support system which had a surface structure and chemistry with less affinity to binding oligonucleotides, or a blocking agent able to adsorb more strongly to the support than the oligonucleotide probe.

The difficulties of non-specific binding were made clearer when a non-radioactive labelling strategy was used for target detection. Most background was probably generated from the non-specific

binding of the HRP labelled oligonucleotide to the resin support. The method used relied on a labelling strategy developed by Urdea et al. (1988). Perhaps by adapting the found assay configuration to one using the support systems (polystyrene and polyvinyl) used by Urdea et al., this difficulty encountered with increased background in association with non-radioactive labelling could be overcome.

4.1 Other and future applications of the sandwich assay and immobilised oligonucleotides

The advantages of an oligonucleotide based sandwich assay may in future facilitate other DNA procedures. This could establish advances both in the development of new laboratory techniques, as well as in the creation of other diagnostic techniques.

Sandwich hybridisations based on random covalent binding of oligonucleotides could be applied in the affinity capture of nucleic-acid sequences, to improve sequence enrichment and purification techniques, of nucleic acids. The advantage of using permanently fixed oligonucleotide capture probes, is that the synthesis of such affinity resins is cheap and also rapid.

Using such "affinity resins" would be an improvement over some of the present methods for both subtractive enrichment and positive sequence selection, where affinity capture is often based on the use of recombinant DNA fragments. The procedure developed by Welcher et al (1986), for example, is based on the affinity of a biotinylated capture probe to cupric-iminodiacetic acid agarose beads, using recombinant DNA probes as capture sequences.

The "affinity resin" would also enable the selective purification of specific nucleic acid sequences from a pool of DNAs, such as the separation of the products generated through PCR

amplification, from the remaining complement of DNA. This would facilitate the characterisation and use of PCR amplification products for further manipulations such as cloning and DNA sequencing. Similarly, selective purification of DNA sequences using oligonucleotides might benefit the characterisation and cloning of infectious organisms, such as viruses.

Hybridisation of a complementary detector probe to the target DNA in control sandwich assays could be used to automatically quantify the efficiency of affinity capture as well as the degree of purity of the final product. As the melting temperature of the hybridised detector probe can be adjusted to be below that of the target DNA, by using long capture probes, such controls could theoretically be performed on a single target sample.

Another application of the immobilised oligonucleotide might be in the sequencing or synthesis of specific target sequences. For this the enzymically immobilised capture probe could be used as a primer, to initiate synthesis of a hybridised target sequence. The sequence specific detector probe again would be useful to determine the efficiency of target capture in control reactions. Using the sandwich assay configuration would, for example, simplify the procedures used for sequence specific cDNA synthesis and the characterisation of specific mRNA sequences.

Alternatively, end-immobilised oligonucleotides could be used for gene assembly. By hybridising oligonucleotides, complementary and overlapping in sequence, first to the immobilised oligonucleotide, then to the next overlapping sequence, a synthetic double stranded gene could be synthesised. With the 5' ends of each oligonucleotide phosphorylated a ligase enzyme could be used to generate two complementary, intact strands of DNA.

Future diagnostic applications of the oligonucleotide sandwich

assay, other than those involving the detection of single base changes, might be in the identification and quantification of invasive viral or bacterial pathogens. In contrast to these human applications, other applications might also include the genotypic analysis and the detection of pathogens in animals and plants.

Commercial interests are directed at producing detection kits which can be used to perform quick and simple tests on samples by the "layman" in a non-laboratory environment. The expected requirements of such tests are that they are as close to 100% reliable as possible and that they are based on a non-radioactive detection system. Production of these tests should also be cheap.

The ideal assay configuration is one in which the test is performed on a filter support system and in which the reaction time is short (as in the "dipstick" arrangement of many existing diagnostic tests).

It seems unlikely that any DNA based assay could ever be expected to be packaged in this way, primarily because DNA is generally contained within cells and not freely circulating as, for example, antibodies and some proteins or metabolic products. What could be hoped for, in future, is a system for DNA diagnosis using automated DNA extractors, synthesizers, amplifiers (for PCR amplification), sequencers and robot arms to perform the routine manipulations involved in analysis. With the application of such instrumentation the oligonucleotide based sandwich assay could become fully automated. It is hoped that this would give a time-saving and commercially viable, DNA based, diagnostic procedure.

4.2 Conclusion

A method has been devised for the rapid detection of single base changes of specific nucleic acid sequences present in purified samples of DNA. The method is based on the adaptation of three methods to produce a novel hybridisation assay:

- 1) Specific oligonucleotide sequences have been immobilised to resin support systems by adapting available immobilisation strategies. These immobilised probes have been used in the affinity capture of target sequences.
- 2) Oligonucleotide detector probes have been employed to detect single base changes in target sequences. The procedures using pairs of oligonucleotide probes, for differential hybridisation to normal and single base mutated target DNA have been utilised. A novel base analogue 2-aminoadenine (Chollet et al., 1988) has been employed to improve the possibilities of differential target DNA analysis.
- 3) By employing PCR amplification, this assay has achieved the sensitivity required for the detection of single copy genes.

As opposed to the typical sandwich assay format (Ranki et al., 1983), the detection process in this system is rapid and is sufficiently sensitive to permit single gene detection in less than 200ng of human genomic DNA. The assay components are easily synthesised and can therefore be adapted to detect other known base mutation sites. Also, the method could prove useful in the affinity capture and detection of a variety of other nucleic acid sequences.

The assay system developed is a novel approach to the detection of single base mutations in human genomic DNA samples.

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